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CULTIVATION OF ANIMAL TISSUES IN VITRO IN NUTRIENTS OF PRECISELY KNOWN CONSTITUTION

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INTRODUCTION

Ever since the inception of the tissue culture method forty years ago the elucidation of the nature of the nutrient medium has presented one of the major unsolved problems. Indeed, the ill-defined character of the nutrient is today one of the chief barriers to a really satisfactory and rational use of the method in the study of physiological questions, for the major theoretical advantage of *in vitro* cultivation should be the possibility which it gives of eliminating the unknowns in the total environmental complex.

The development of any technique represents a two-pronged problem. One must *get results*, and, if they are to be fully useful, one must be able to say *by what means those results were obtained*. Harrison got results, in 1907, using clotted lymph as his nutrient (47). Burrows improved on those results by substituting plasma for lymph (20), and Carrel by adding embryo juice to the nutrient (26). But there the matter has stood, and although Lewis (53), Lewis and Lewis (54), Drew (35), Baker (6, 7), Baker and Carrel (8, 9, 11-13), Baker and Ebeling (14), Vogelaar and Erlichman (90-93), Simms and Stillman (81-83), and others, have replaced one or more of the unknown ingredients with partly or wholly synthetic equivalents, there is still today no nutrient described in the literature capable of supporting continued growth or even long continued survival of animal tissues which does not contain plasma, serum, bouillon, peptone, fibrin digest or tissue extract in some form. No satisfactory nutrient capable of precise definition is now available.

The establishment of a method may be a purely pragmatic, empirical matter, as Harrison, Burrows and Carrel and their followers have shown. But the precision of that method must result from experi-

ment, often of the most extensive and rigorous sort. Haberlandt, when he first defined the concept of tissue cultures, clearly recognized this fact (44). He assumed that this preliminary empirical step would be as simple in the case of plant tissues as it subsequently proved to be for animal tissues. He therefore emphasized the second, experimental approach. In this assumption, however, he proved so thoroughly mistaken that twenty-four years later (51) a distinguished colleague could publicly and in all seriousness pronounce the problem "hopeless" (aussichtslos). But Küster was also mistaken and the last fifteen years have seen a rather satisfactory answer to this "hopeless" problem. It is the author's conviction that the corresponding problem, as regards animal tissue cultures, is equally amenable to experimental solution. It is with this conviction, and with the background of my rôle in the solution of the plant tissue culture problem, that the problem of growing animal tissues in nutrients of known and reproducible constitution, that is to say synthetic nutrients, has recently been approached, albeit with some hesitation.

The attack upon such a problem requires at the outset certain arbitrary decisions, which will be based on such store of accumulated fact as we may possess. In surveying the plant problem (1924-1928) I very early discarded on theoretical grounds most of the tissues which had been tried before, and chose three: young embryos, seed primordia, and root tips, as most suited for manipulation (95). Preliminary experiments quickly eliminated the first two (96). A single species of plant was early chosen for concentrated study. The flask-culture method of Robbins (71), the inorganic nutrient solution of Uspenski and Uspenskaja (89) and the use of yeast (Robbins, 72) but *not* of autolyzed yeast, were chosen as a basis on which to build. The methods developed from such a beginning are now applicable to a great variety of plant tissues and organs, of many different species of plants, and under a considerable range of different technical procedures (110-112, 116). Approach to the problem of cultivating animal tissues requires a similar series of arbitrary choices as regards *1*) tissues and animals best suited for investigation, *2*) technical procedures capable of furnishing results without too great complications or cost, and *3*) last and most important, nutrients with which to begin.

Choice of Tissues and Tissue Sources: Carrel and Ebeling's work on the effect of age of tissue source, and plasma source (Baker and Carrel, 10; Carrel and Ebeling, 30, 31) on rate of growth *in vitro*

has led to the general belief that embryonic tissues respond more satisfactorily to *in vitro* cultivation than do adult tissues. This concept is now under some question (Doljanski and Hoffman, 33; Hoffman and Doljanski, 49). The most readily available, cheapest and most easily handled vertebrate embryos are those of the chick. As a result of these two facts chick embryo tissues have been more extensively studied than any others and a very considerable chick embryo tissue culture lore has grown up. These constitute a valid set of reasons for choosing chick embryo tissues for preliminary work on any problem of this sort. It is recognized that investigation should pass on to mammalian tissues and especially to tissues of adult mammals just as soon as adequate procedures are available. The decision as regards specific tissues is less simple. Most of the literature deals with the so-called "fibroblasts" which in the standard Carrel technique tend to over-run all other types of cells. The visual criteria of physiological health or illness in such cells (and this is also true of epithelium, leucocytes, chondrioblasts, neuroblasts and most other cell types) are the rather indefinite criteria of rapidity of migration, mitotic index, degree of fatty degeneration, phagocytosis, and so on. It is well known that migration rate may bear no relation or even an inverse relation to increase in mass, that fatty degeneration is only a *late* stage of "illness", that the mitotic index of such pathological cell types as neoplasia may be much higher than that of healthy cells. A more reliable criterion for judging physiological condition is certainly desirable. Two such criteria do seem to be available. Muscle has not been as extensively cultivated *in vitro* as have some other cell types but enough has been done to show that in complex media rhythmic contraction *can* be maintained for long periods. The longest periods seem to be Olivo's (63, 64) report of six months for cultures of heart tissue from 3-day chick embryos in a plasma-embryo juice medium and an equal period for 8-day chick embryo heart muscle in an embryo-juice Locke solution, reported by Hogue (50). Here is obviously an excellent visual sign. Pogogeff and Murray (69) have recently reported rhythmic contraction in skeletal muscle *in vitro*. A heart muscle which is beating strongly at a steady rhythm of 60 to 100 contractions per minute is certainly in good condition. If the rate is excessively slow, is shallow in extent, is irregular or is excessively rapid (fibrillation, 150 to 500 contractions per minute) one can immediately assume that the condition is in some respect unsatis-

factory. This, then, is a clear-cut visual diagnostic criterion of *condition of survival* which bears no necessary relation to growth. Growth, on the other hand, can be judged visually, at least in cultures of fibroblasts and to some extent in those of epithelium, on the basis of *establishment of new centers of cell accumulation.* If, in a flat sheet of cells either migrating or growing out on the substratum, or at isolated points on the substratum beyond the margin of continuous growth, there arise aggregations of cells which pile up into three-dimensional masses, constituting new growth centers, we can say with considerable conviction that the culture is *growing satisfactorily* without attempting to measure increment in terms of increase in area, dry weight or other quantitative criteria. Such *qualitative* measures are essential in a type of work such as that here contemplated, where it will be necessary to study a great number of environmental permutations. It would be quite impossible to use strictly quantitative criteria on a sufficiently large number of cultures.

With these facts in mind it was decided in the preliminary work to use heart muscle and skeletal muscle (including considerable amounts of connective tissue) from chick embryos. Eight-day embryos proved quite satisfactory. These observations were later supplemented with studies on other tissues.

Choice of Technical Procedures: The technical procedures at present available for animal tissue cultures may be divided into four general groups: *1*) hanging drop cultures, which includes the standard method of Harrison, (47), Burrows (20), Carrel (26) and Lewis and Lewis (54) and the "sitting-drop" method used by Gey and Gey (43); *2*) the open watch glass method of Fell (37) including Carrel's Gabrischewski glass method (25) and Maximow's method (62); *3*) the closed vessel method of Carrel (Carrel flasks) (27); and *4*) the roller-tube method of Gey (42, 43) and Lewis (56), including that of Shaw, Kingsland and Brues (80).

1. The hanging drop method is the best of all for minute high-power observation of the behavior of individual cells, and when nutrient is changed every 48 hours permits prolonged cultivation under approximately optimal conditions. The small size of the nutrient drop, however, precludes accurate control of the chemical or physico-chemical changes which the nutrient undergoes. Moreover, the method requires an enormous amount of manual manipulation per culture so that it is quite impossible to maintain even moderately large num-

bers of cultures without a great deal of technical assistance. It is thus not well adapted to nutrient studies and should be used only as a secondary method for special purposes.

2. The open watch glass method is very simple, technically, and permits both the use of large masses of tissue and large volumes of nutrient. The conditions of gas exchange and control of gas supply are, however, poor and the method does not permit microscopic examination at distances of less than two or three millimeters, unless one wants either to go through the time-consuming procedure of isolating special mounts for examination or else run the risk of contamination involved in observation at the open liquid or semi-solid surface. Although excellent for certain types of metabolic studies (Fell and Robison, 38), it thus has serious disadvantages where one wishes to follow the detailed morphological behavior of a tissue over long periods.

3. The Carrel flask method permits the use of large tissue and nutrient volumes under accurately controlled nutrient conditions and can be adapted for excellent control of gas-exchange. It also permits observation under any desired magnification. Where financial and labor considerations are not important it is an excellent method. The flasks, however, are expensive, bulky, difficult to clean, somewhat clumsy to handle, and do not permit adequate exchange of nutrient except at the expense of time-consuming manipulations. This method therefore is also far from ideal for extensive studies.

4. The Gey-Lewis roller-tube method involves the use of standard rimless 16 x 150 mm. test tubes of Pyrex glass which are cheap, easily handled and easily cleaned. Each tube can contain as many as 30 separate cultures so that a series of 4 or 5 tubes is equivalent to 50 to 100 hanging drop cultures or a dozen or more Carrel flasks. The nutrient volume (1 or 2 ml.) is twice that commonly supplied in the Carrel flask. The gas phase is about one and one-half times as great as in the Carrel D 3.5 flask. Yet because of their shape these tubes occupy far less space and are much more easily handled than are flasks. Since the tubes are rotated constantly there is a continuous change of nutrient at the cell surface, neither supply of food constituents nor removal of metabolic products depending on diffusion as they do in the static, semi-solid nutrient commonly used in Carrel flasks, and the cultures are exposed directly to the gaseous phase through some 90 per cent. of each rotational cycle. Moreover, as we

shall see later, the manipulations required in setting up cultures and in changing nutrients can be very greatly simplified as compared with the procedures now generally considered necessary. Cultures can be examined at will under the low-power of the compound microscope (mag. x 100). If tubes are specially selected for thinness of wall (0.3 mm.) it is possible to use the high-power (x 200). The only serious defect in the method is the curvature of the field which interferes with obtaining good photographs of large areas of tissue, and the constant motion which precludes the use of time-lapse cinematography. These objections are far outweighed by the advantages. With tissues already isolated and nutrients ready an operator with a single assistant can easily set up 50 tubes (500 to 1000 cultures) in a half-hour. For nutrient studies where large numbers of comparable cultures are necessary the roller-tube method seems to be far and away the best method now available. It has therefore been chosen for all the preliminary work on this problem.

Choice of Nutrients: The objective of the present study is the development of *nutrients all of whose components are precisely known both as to quantity and as to chemical and physico-chemical composition,* which shall be capable of supporting potentially unlimited survival and/or growth of the tissues in question. No such nutrients are known today. Nutrients of known composition are to be found in the literature but they will support only limited survival of tissues. The solutions of Ringer (70), Tyrode (88), Locke (57), Pannett and Compton (68), and others, are examples. Only that of Ringer seems to have been evolved by a process of careful and logical experimentation (70) and that was developed nearly 60 years ago. Nutrients capable of supporting unlimited growth are also known but they are all complexes of essentially unknown composition containing as they do, in every case, peptone, tissue juice, plasma, serum, broth, fibrin digests, bacterial products or some other organic complex.

The problem of developing synthetic nutrients may be approached in either or both of two ways: By synthesis, that is by combining known materials suspected of being important for growth, in various combinations, retaining combinations that are an improvement over some arbitrarily chosen and only partially complete basic solution, discarding unsatisfactory combinations and hoping thus to build toward a complete nutrient. With this method one always faces the risk that the limiting factor at any given time will remain unrecog-

nized. The real significance of a particular experimental variable cannot then be detected and negative results may lead the investigator to discard really important facts. The other is to analyze one or more of the adequate but complex nutrients, gradually eliminating the unessential components of the complex until the residue is completely known. A combination of the two approaches was employed in solving the similar problem as regards nutrients for plant tissue cultures (97-109, 113-115). Carrel and Baker have used the second method in approaching the animal tissue nutrient problem without notable success (Baker 5-7; Baker and Carrel, 8, 9, 11-13; Carrel, 26-28; Carrel and Baker, 29; Carrel and Ebeling, 30, 31). Aside from this work of Carrel and Baker, some studies of Drew (35), Fischer and Demuth (41), Vogelaar and Erlichman (90-93), and Simms and Stillman (81-83) there is very little pertinent literature available.

The analytic method presents such manifold difficulties and has to date resulted in so little progress that it has not seemed worthwhile to use it extensively in the present study. There has accumulated, however, in the past twenty years a very considerable amount of information on a number of nutrient factors involved in growth which has not, to my knowledge, been taken into consideration sufficiently in any of the attempts to develop "synthetic" nutrients. I refer particularly to our knowledge of the importance of metallic trace elements, amino acids, vitamins and hormones. It seems probable that a great deal can be done by a judicious use of this knowledge.

The nutrient portion of the environment for any cell or tissue must include certain materials and characteristics which are universally recognized but have not always been given adequate consideration. Maintenance of life of animal cells depends on the provision, under properly balanced conditions of *1*) total osmotic value, *2*) pH, and *3*) gas exchange, of *4*) a supporting substratum, *5*) water, as a protoplasmic constituent and as a solvent carrier for other essential materials, *6*) inorganic salts, both the "nutritive" salts—chlorides, sulfates, nitrates, phosphates, carbonates and possibly citrates, malates and pyruvates of sodium, calcium, potassium and magnesium, and the "accessory" or "oligodynamic" salts containing iron, manganese, zinc, iodine, bromine, fluorine, silicon, copper and perhaps other elements such as cobalt which presumably have a catalytic function, *7*) organic building blocks, especially nitrogenous substances such as amino acids and their combined products and possibly glucosides, nucleic acids,

etc., *8*) energy sources in the form of combustible materials of some sort, especially carbohydrates but also possibly such carbon compounds as the citrates, malates and pyruvates (respiratory intermediates), *9*) vitamins, hormones and other essential organic catalysts, and lastly *10*) some means of carrying away and eliminating waste products. Each of these factors will be examined in detail though not necessarily in the order given here.

Equipment and Technical Procedures

As has been said above, the roller-tube method has been chosen as best suited for the study of large numbers of cultures. Löwenstädt described a roller-tube method in 1925 (59). Such a method is also mentioned by Parker (67, p. 118) as having been used by Carrel in 1929 but seems not to have been published. Fell used a fixed test tube method in 1928 (37) with some success. None of these methods was established sufficiently to be widely used. In 1933 Gey published a brief description (42) of a roller-tube method which he (43), Lewis (56), and Coman and Stabler (32) subsequently elaborated and which is now used with considerable success in several different laboratories. The author has seen this method in use in Baltimore and in Philadelphia where it has proved very satisfactory. The descriptions published to date, however, are not sufficiently explicit so that a novice could build and use the apparatus without further information. The method is capable of rendering such important service to biological science that it seems worthwhile to give, here, a fairly detailed description of the equipment and procedures as I have adapted them for use on this problem.

The principle is simple. A number of tissue fragments are attached by some means to the inner surface of a 16 x 150 mm. Pyrex lipless test tube. The tube is charged with 1-2 ml. of a liquid nutrient and stoppered with a number 0 rubber stopper. It is then placed in a carrier which holds it at such a slope that the nutrient comes to about 30-40 mm. from the end of the stopper and which rotates it slowly around its long axis. This causes the nutrient to flow across the tissue, the latter being immersed from 5 to 20 per cent. of each cycle, depending on its position in the tube, and exposed to the gaseous phase through the other 80 to 95 per cent. of the time. The whole is enclosed in an incubator. The mechanisms for obtaining these results may be elaborate, or they may be extremely simple.

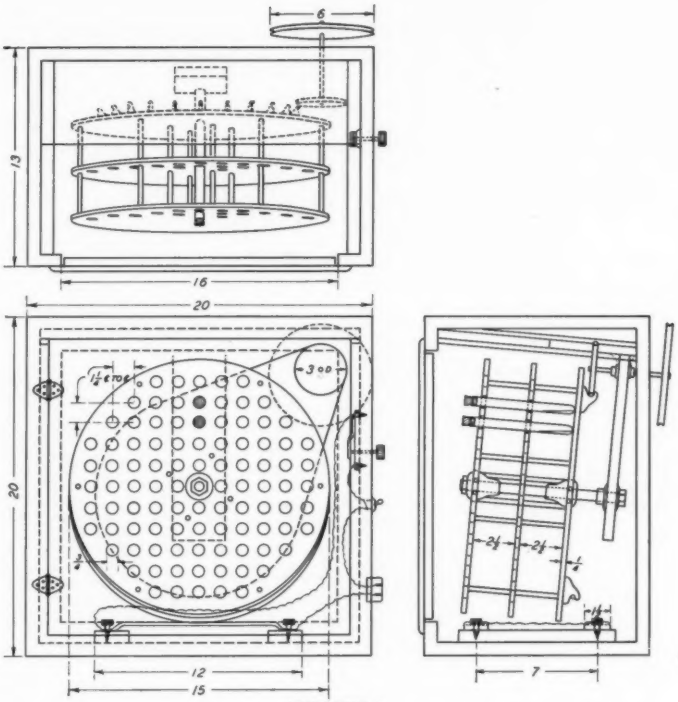

FIGURE 1
Working drawing of rotor and incubator. (Drawing by J. A. Carlile.)

The equipment developed for the work in this laboratory consists of two incubators. Each is 27 inches high, 20 inches wide and 21 inches deep, outside measurements. They are made of ¾-inch "Celotex" covered with ¼-inch plywood. Each has a removable front, pierced by a 16 x 16-inch door. Each is heated by two 12 x 1¼-inch bar-type resistance units (Westinghouse span heater, 220 watt, 115 volt. $2.10) connected in series and controlled by a bimetallic thermoregulator (type T-22 breaker, Lyon Rural Electric Co., San Diego, Cal., $1.65). The right-hand incubator has a 6 x 11-inch door on the left side, back, which opens on three drawers in each of which a dozen and a half eggs can be placed for incubation. The eggs are stood upright, air-sac up, on racks made by boring a series of 1½-inch holes

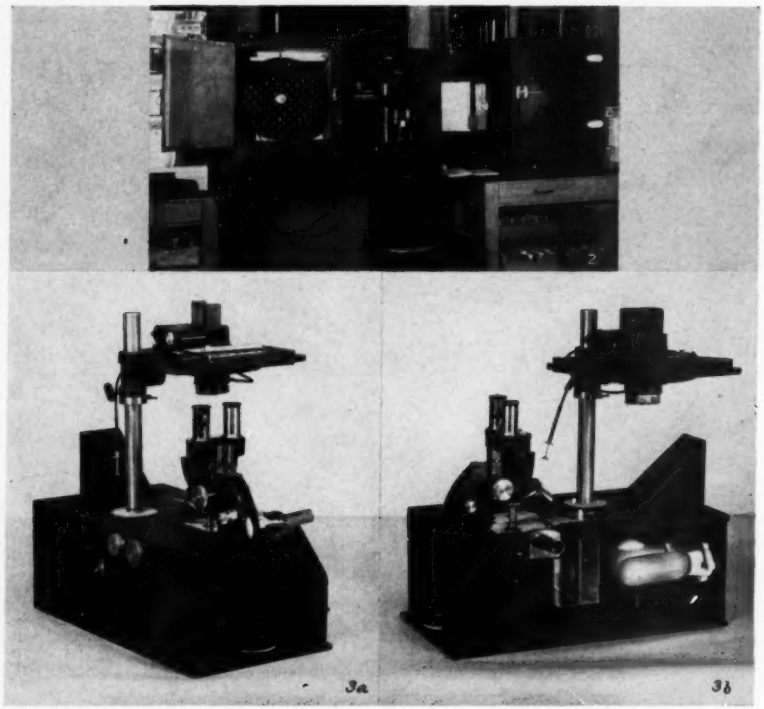

FIGURE 2

Roller-tube rotors in their incubators, microscope in its warm-box, and camera. These incubators are somewhat higher and deeper than the simple type illustrated in Figure 1. (Photograph by J. A. Carlile.)

FIGURE 3

Warm-box with microscope and camera. *A*, left-hand side, showing control knobs of mechanical stage, switch and thermoregulator knob. *B*, right-hand side, with door removed to show carrier tube of mechanical stage into which culture tubes are inserted, water cell for cooling light, photoenlarger bulb and heating bulb. (Photographs by J A. Carlile.)

in pieces of one-inch board placed in the bottom of each drawer. Eggs should *not* be turned. Each incubator is provided with a rotor capable of carrying 100 tubes. The rotor is made from three disks of plywood 15 inches in diameter. Two of these are pierced with ¾-inch holes placed 1¼ inches center-to-center each way, on a square pattern. This gives 5 rows of 11 holes each, the center hole being

covered by the wheel hub (54 holes), 4 rows of 9 each (36 holes) and 2 rows of 5 each (10 holes), totaling 100. The third disk is left solid. These disks are supported by 10 dowels, 6 near the outer rim and 4 near the center, of such a length that the disks lie 2½ inches apart. They are suspended on bearings made by sawing a bicycle front-wheel hub in two, screwing the halves to the outer and inner disks and connecting them with a length of ⅝-inch pipe soldered to the sawed faces. The axle consists of a 5/16-inch rod threaded to take the chucks of the wheel bearings and fixed rigidly at one end into a "T"-shaped wooden support which can be slid into a pair of grooved channels screwed into the top of the side walls of the incubator, at such an angle as to provide the proper slope for the tubes. A series of brackets are screwed to the back of the third (solid) disk in a 12-inch circle so as to provide a pulley on which to run a sewing machine belt. A second ball-bearing axle, carrying a 3-inch pulley at its inner end placed in line with the pulley on the rotor and with a 6-inch pulley on its other end where it projects through the back of the incubator, is attached to a strong support at the bottom of the incubator. From these pulleys belts are run to a 2-inch double pulley activated by a 1:100 reduction gear (Boston Gear Works, Inc., 151 Lafayette Street, New York, New York, no. LA 100 parallel drive reductor, $9.00) which is in turn driven by a 1/16 H.P. 1500 R.P.M. electric motor. The motor is controlled by a rheostat and when driven at minimum speed gives a rotation speed at the tubes of about one cycle every 7 minutes. White lines are painted between the rows of holes in the rotors, to facilitate finding any desired set of cultures. This is especially important when rotors are nearly or quite full since without them one is likely to examine rows running perpendicular to the order in which tubes have been introduced. In inserting tubes it is important to keep them evenly distributed on the rotor, for any serious imbalance will set up considerable torque which may stop the wheel, causing the belts to slip.

The two incubators together cost about $100.00, fully equipped including motor. They are, of course, much more elaborate than is absolutely essential. Dr. Lewis has made excellent small rotors of heavy Bristol board, bearings taken from roller skates, driven by stripped-down self-starting electric clocks (a drive pulley can be attached to the stem of the sweep second hand) in homemade boxes heated with electric globes, which can be built for about $15.00 and

are quite adequate for small scale work. Dr. Gey's equipment is more elaborate than mine. In my own laboratory the two incubators are set on a table about three feet apart with the drive motor between and against the left-hand incubator. The microscope, in its warm-box and equipped with camera for photomicrographs, is placed between the two boxes, likewise somewhat to the left of center, leaving room for a notebook between it and the right-hand box. In this position any tube in either box can be reached without leaving the chair in front of the microscope.

Rapid cooling of the tubes causes condensation and may injure cultures although they have been found to be surprisingly resistant to considerable variations in temperature. To obviate this, it is best to keep the tubes reasonably warm while under observation. It is also desirable that they be supported at about the same slope as that used in the rotor. Dr. Lewis furnishes support by soldering two brass plates 180 x 10 x 2 mm. to three cross bars in such a way that when placed on the microscope stage one end of the narrow plates will be raised 10 mm. above the other. This is placed on the microscope stage and the culture tube laid between the slides on which it can be moved longitudinally or rotated at will. The same result can be obtained with a block of wood 7 x 1 x 1 inches, bored lengthwise with an 11/16-inch hole at the desired slope and cut out, top and bottom, to provide visibility. This is somewhat less stable than the heavier metallic support. For work in this laboratory a specially designed mechanical stage was built providing longitudinal motion calibrated in mm. and rotary motion calibrated in degrees. This permits accurate re-location of any desired culture on consecutive days, a matter of some importance when serial photographs are to be taken. Heat may be provided by a cardboard box containing an electric light globe and placed, face forward, behind the miscroscope so as to reflect light and heat onto the stage (Dr. Lewis' method). A more elaborate box 10 inches wide, 6 inches high and 16 inches long is used here. Removable panels at the front allow the microscope to be inserted and a door on the right-hand side provides access to the condenser and other equipment. The top is cut out to allow manipulation of the mechanical stage which is, however, covered with a thick sheet of cellophane pierced by a 15 mm. hole through which the microscope objective passes. In this way a closed chamber capable of excellent temperature control is provided. Illumination sufficient for photomicrographs at

exposures to 1/10th second is provided by a 250 watt photoenlarger bulb, placed at the back of the box. More concentrated light sources provided with suitable condensers must be used for shorter exposures. Excess heat is carried off by a chimney above the light. Heat rays are filtered out by a 10 x 10 x 3 cm. water cell and illumination for visual work is reduced by means of neutral filters. This light is controlled by a switch on the left-hand side of the box. Additional

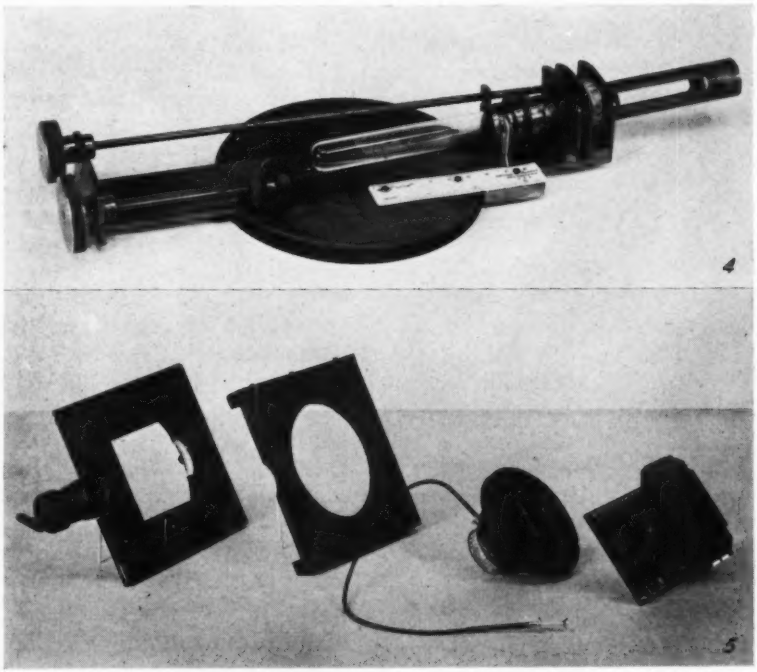

FIGURE 4

Mechanical stage providing lateral motion calibrated in millimeters and rotational motion calibrated in degrees, maintaining the culture tube at the same slope as is provided in the incubator (Figures 1 and 2). (Photograph by J. A. Carlile.)

FIGURE 5

Camera assembly showing bracket providing lateral horizontal motion (in an arc around stand), slide providing frontal horizontal motion, shutter carrier providing rotation around the optical axis, and camera with clear, ruled glass for focusing. (Photograph by J. A. Carlile.)

controllable heat is provided by a single carbon-filament bulb wired to a thermoregulator of the same type as that used in the incubators and placed in the box on a level with the miscroscope stage. The temperature was adjusted by placing a thermometer inside a standard culture tube on the microscope and setting the thermoregulator to break at 37° C..

The photomicrographs used in this paper were all made with a camera devised to provide quick and easy manipulation at low cost (Figs. 2, 4, 5). A "Bantam Kodachrome Adapter B" was built into a wooden carrier which permitted rotation in the horizontal plane and back-and-forth motion of about 1¼ inches. This was suspended on a metal bracket which could be adjusted to the proper height with a ring-clamp on an upright support and could be swung around this support to give right-and-left motion and to get the camera out of the way when not in use. A shutter (Wollensak) providing time, bulb, and 1, ½, 1/5, 1/25, 1/50 and 1/100th second exposures and a Bausch and Lomb focusing lens completed the outfit. The whole cost $54.27. With Kodak PX828 plus X film pictures can be taken at a cost of about three cents each, which will stand enlargement to 3 to 5 times their initial size. It has been found best to change from binocular to monocular tube when taking pictures, but this shift can be made, a picture taken, and the tube shifted back to the binocular in less than a minute's time from the time a suitable subject is located, with results equal to those obtainable with much more expensive and complex apparatus.

One other piece of special equipment has been found useful in setting up cultures. A piece of plate glass 14 x 20 inches is fixed in a frame so as to hold it horizontal, 7 inches above the table (Fig. 6, left). The frame is so built as to be open at the front and through the front 12 inches of the sides. Placed on the table with the forward edge projecting about 2 inches beyond the edge of the table, this furnishes a shield under which dissections can be made with very little danger of contamination from falling dust or from the operator's breath, yet gives full visibility and almost complete freedom of movement.

The procedure in making chick embryo cultures is as follows. The equipment required consists of three pairs of fine forceps, one straight and two curved; three short-handled needles, one straight, one with a hook about 4 mm. diameter of curvature and one with a right-angle,

sharpened blade tip about 5 mm. long; two pairs of small curved-bladed scissors; a one liter stainless steel beaker with immersion heater, in which is suspended a 100 ml. beaker provided with a rubber disk in the bottom—the instruments can be kept boiling with their points in this beaker when not in use; a wire rack placed under the dissecting shield on which instruments can be supported; three pairs of sterile Columbia-type shallow watch glasses; twelve U. S. Bureau of Plant Industry small type Syracuse watch glasses in a Petri dish, likewise sterile; a micro-Bunsen burner; a short piece of ½-inch iron rod; and pipettes with which to transfer tissue to the culture tubes. Culture tubes closed with cotton plugs, stoppers, pipettes and glassware are sterilized beforehand. Rubber stoppers can be sterilized in Petri dishes, separated from the glass by disks of waxed weighing paper to prevent their sticking to the glass. The dishes are wrapped in paper and sterilized in the autoclave at 1 atm. for 20 minutes, followed by 20 minutes under vacuum to dry them thoroughly. Glassware (pipettes, watch glasses, tubes, etc.) are sterilized by dry heat. A temperature of 120° C. for 2 hours or more, preferably overnight, has been found adequate.

The dissecting shield is placed on the table in the culture room, the front edge of the glass projecting an inch or two beyond the table edge. The wire rack is placed under it, toward the back. A Petri dish with a dozen sterile U. S. Bureau of Plant Industry watch glasses is placed at the left. The beaker, boiling and carrying the needles, forceps and scissors is placed at the right. Three or more pairs of Columbia watch glasses are opened and placed under the shield, one glass serving as cover for each member of a pair. A sterile U. S. Bureau of Plant Industry watch glass is placed beside them. A series of candled eggs are placed on end, air-sac up, in 50 ml. beakers, each in a half Petri dish. The three pairs of forceps are taken from the beaker and stood against the wire rack to cool. With a bit of absorbent cotton the top of an egg is sterilized with 85 per cent. alcohol. It is cracked all around with the end of the iron rod, swabbed off again with alcohol and placed under the shield. The straight forceps are jabbed into the egg near the base of the air-sac and the egg turned with the left hand so that the top is chipped off. This is removed and discarded, and the straight forceps returned to the beaker. With a pair of curved forceps the membrane is broken and pulled aside. If the eggs have been incubated on end the embryo will be against

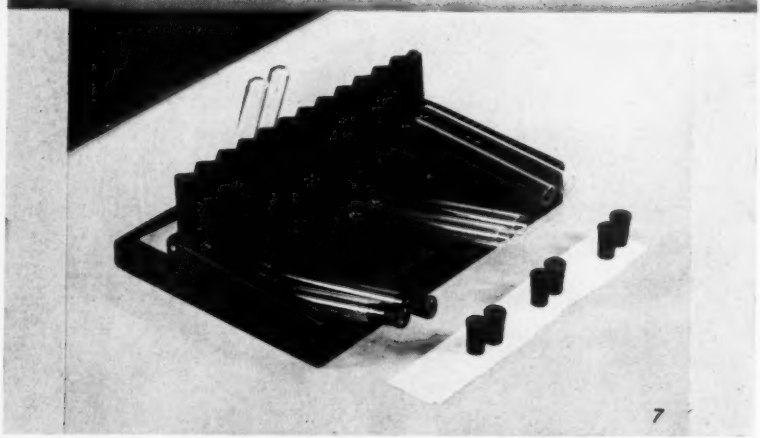

FIGURE 6

Equipment used in making tissue cultures. Reading from left to right: Petri dish containing sterile U. S. Bureau of Plant Industry Syracuse watch glasses; glass protective shield under which dissections are carried out covering, at the front, one micro-Syracuse watch glass, two pairs of Columbia watch glasses, at the rear a wire rack carrying three pairs of forceps, a culture pipette, and a pair of curved scissors; an egg placed on end in a 50 ml. beaker and Petri dish, and covered with alcohol-soaked cotton; an iron rod used in opening eggs; a micro-Bunsen burner; a 1-liter beaker with heater and suspended 100 ml. beaker containing dissecting needles being boiled; alcohol; a 100 ml. beaker containing stoppered tube of sterile nutrient and culture pipette; and a rack with seven culture tubes. (Photograph by J. A. Carlile.)

FIGURE 7

Renewing nutrient. Tubes are taken from the incubator and placed as in the foreground. After the stoppers and mouths of the tubes have been thoroughly swabbed with alcohol, the stoppers are removed and placed on a strip of sterile filter paper at the front, while the tubes are sloped at the back so as to drain on a second strip of filter paper. Fresh nutrient is pipetted into the tubes which are then sloped as in the right-center until they are ready to be re-plugged. The tubes are stoppered, the stoppers and tube mouths are washed with warm water and the tubes returned to the incubator. (Photograph by J. A. Carlile.)

or very close to the air-sac. With forceps in the left hand and the curved needle in the right, the amniotic membrane with the embryo is lifted and the needle hooked under the neck. The membranes are then carefully pulled away, the embryo freed and transferred to a U. S. Bureau of Plant Industry watch glass. The neck is severed with the forceps, the head discarded, the egg set aside and the needle and forceps returned to the sterilizing beaker. With the straight needle in the left hand and the right-angled one in the right, the embryo is placed on its back, an incision made from belly to neck and the heart removed on a needle and transferred to a covered Columbia watch glass. The embryo is then turned on its belly, an incision made along the backbone and the skin peeled back left and right so that it is everted over the legs. With curved forceps the lower portion of each leg is removed and, with the adhering skin, discarded. The legs are then severed at the thigh and placed in a second watch glass. Here the flesh is dissected off the bones and placed in a third covered dish. If material is desired for nerve cultures this is best obtained from the spine where the spinal ganglia provide excellent growth of neural processes (see later). Pieces are snipped out with fine forceps from the middle of the back for a distance of 3 to 4 mm. toward the tail. These are placed in a fourth dish. The remainder of the embryo is then discarded. This process is repeated until all eggs to be used have been opened. For 5- to 8-day embryos it is best to use a dozen eggs. Larger embryos permit the use of correspondingly smaller numbers but may require larger dishes in which to carry out the dissection.

After the tissues are all dissected, the contents of each dish is triturated thoroughly with a sharp pair of curved scissors or with a pair of sharp scalpels or cataract knives until no pieces remain more than 0.3 mm. in diameter. Then, without using any diluent, the tissue *brei* is taken up in a pipette having a curved tip. Up to this point the operation can be performed satisfactorily by a single person. At this point it is helpful to have an assistant hand each tube to the operator after removing its cotton plug. The tissue *brei* is "streaked" along one side of each tube, beginning at the tip and coming up about 70 mm. toward the mouth. The tubes are re-plugged and set in a sloped rack, tissue streak up, as fast as completed. In practice I have sometimes charged tubes with heart and leg muscle in separate tubes, sometimes with both mixed in the same pipette, depending on the purpose. When all tubes have been "streaked" they are divided into

as many equal groups as there are separate nutrients to be tested. It is best to use not fewer than 5 tubes (50 to 100 cultures) to each nutrient. Four, five or six nutrients at a time makes a satisfactory series (20 to 30 tubes). Nutrient, previously prepared, is taken up in a 10 ml. pipette and 2 ml. placed in each tube. The Bunsen burner is then lighted, rubber stoppers are taken from their Petri dish and the cotton plugs replaced therewith, flaming the mouth of each tube briefly before inserting the stopper. The tubes are set aside for about 2 hours to "set", so that the tissue fragments dry onto the glass, before being placed in the incubator. If this is not done most of the tissue may wash loose.

It will be noted in this description that nothing is said about flaming instruments or tubes except at the final stage. This was intentional. It has been the author's experience in many years of work with plant tissues, fully confirmed with animal tissues, that much of the routine commonly recommended by bacteriologists and by experimental microbiologists in general, for the maintenance of asepsis, is quite unnecessary and only serves to reduce the efficiency and, hence, success of the work. This is especially true of the practice of flaming. At no point is this *necessary* and only in handling the stoppers does it seem even desirable. If glassware is sterile to begin with, instruments are thoroughly boiled, and tissues or eggs are given an external swabbing with alcohol and are then *opened carefully,* contaminations will seldom occur. Not one per cent. of this laboratory's cultures develop contaminations. *Theoretically perfect* asepsis may be a medical "must" for psychological reasons, justifying the use of many practices in the hospital operating room. This may also be true in the handling of tumor materials which so often carry secondary invaders, but in the experimental laboratory these are commonly only encumbrances. The wearing of special masks, gowns, gloves, etc., the practice of "scrubbing up", the use of flamed instruments, the prohibition against blowing into pipettes (in spite of their cotton plugs) and similar customs only distract attention from the real problem of *rapid, precise and clean manipulation under reasonable operating conditions.*

Nutrients

The nutrient used in this work has a constitution so different from that of any other now in the literature that it cannot be considered

a modification of any of these. The means by which it was developed will be discussed later. In actual procedure nutrients were made up in nine stock solutions, each having a concentration twenty times that desired in the final nutrient. These were as follows:

1. Dextrose 17 g.
   H_2O 100 ml.
   Sterilize by autoclaving.

2. NaCl 14.00 g.
   KCl 0.75 g.
   $Ca(NO_3)_2 . H_2O$ 0.42 g.
   H_2O 100 ml.
   Sterilize by autoclaving.

3. $MgSO_4$ 0.55 g.
   H_2O 100 ml.
   Sterilize by autoclaving.

4. $Fe(NO_3)_3 . 9H_2O$ 2.8 mg.
   H_2O 100 ml.
   Sterilize by autoclaving.

5. Amino acids:

A.	dl-lysine-HCl	312 mg.
	dl-methionine	260 mg.
	dl-threonine	260 mg.
	dl-valine	260 mg.
	l-arginine-HCl	156 mg.
	l-histidine-HCl	52 mg.
	phenol red	20 mg.
	H_2O	25 ml.
B.	dl-isoleucine	208 mg.
	dl-phenyl-alanine	100 mg.
	H_2O	25 ml.
C.	l-leucine	312 mg.
	dl-tryptophane	80 mg.
	H_2O	50 ml.

The five amino acids in group A go into solution readily and can be dissolved first in 25 ml. of water. When fully dissolved they can be added to the dry amino acids of group B, which dissolve less readily, shaken and then 25 ml. more water added. Shake until dissolved. The two amino acids in group C wet with difficulty and do not dissolve readily. They should be wetted under vacuum with 50 ml. of water, then heated, shaking constantly, and the combined solutions A and B finally added. After solution is complete NaOH should be added to bring the pH to about 7.6 as estimated from the phenol red color, the mixture distributed to tubes and autoclaved. There is no evidence that autoclaving injures this solution in the least.

6. B vitamins:

thiamin	2.0 mg.	ca-pantothenate	2.0 mg.
pyridoxine	10.0 mg.	biotin	2.0 mg.
niacin	10.0 mg.	choline-HCl (= 0.1 ml.)	100.0 mg.
riboflavin	2.0 mg.	folic acid	0.02 mg.
inositol	10.0 mg.	H_2O	1000 ml.

This can be autoclaved or filtered. While autoclaving is generally supposed to destroy thiamin it has been my experience with nutrients for plant tissues (103, 106, 112, 115), confirmed by others (16, 18, 73, 77, 78) that autoclaving does *not* destroy the effectiveness of such solutions, attributable to thiamin activity. Possibly the thiamin may be broken down only to its constituent thiazole and pyrimidine portions, and these either separately or in combination are the effective agents (Robbins and Bartley, 74; Bonner, 18). There is, however, another possibility. Thiamin is usually added to plant tissue nutrients at a concentration of 10^{-7}. The limiting measurable concentration by bio-assay with plant tissues is about 10^{-11}. Destruction of all but 1/10,000th of the introduced thiamin by autoclaving would thus still leave a detectable though suboptimal level of "thiamin activity". In practice it has therefore seemed permissible to autoclave this solution in spite of the theoretical objections to such a procedure.

7. Vitamins other than the B group:

carotene (90% α, 10% β)	2.0 mg.
vitamin A	2.0 mg.
ascorbic acid (vitamin C)	10.0 mg.
glutathione	20.0 mg.
cysteine-HCl	20.0 mg.
phenol red	10.0 mg.
H_2O	1000 ml.

Add NaOH until a pH of about 7.6 is obtained. Filter and set aside in stoppered tubes.

8. Phosphate buffer:

$Na_2HPO_4 . 12H_2O$	290 mg.
KH_2PO_4	52 mg.
H_2O	100 ml.

Sterilize by autoclaving.

9. Sodium bicarbonate:

$NaHCO_3$	1.100 g.
H_2O	100 ml.

Dissolve, bubble CO_2 through until thoroughly saturated so that all carbonate may be transformed into bicarbonate, then filter into tubes which are immediately stoppered with rubber.

10. Distilled water. Autoclave.

A convenient amount of nutrient is 40 ml.. This is made by taking 2 ml. each of solutions numbers 1 to 7, inclusive, then adding 22 ml. of sterile distilled water and finally adding the phosphate and bicarbonate. These last two must be added after the water or precipitates will form which cannot easily be re-dissolved. The final solution will have a pH of about 7.4 and will keep indefinitely in the refrigerator if tightly stoppered. If left unstoppered it rapidly loses CO_2 and becomes too alkaline.

Controls

In most experimental studies of biological materials it is generally considered necessary to have an arbitrary basis of comparison designated as a "control". In physics, chemistry and mathematics controls are not used since the mere omission of a procedure gives a result well enough known so that it is unnecessary to set it up for each experiment in turn. In animal tissue culture work in the past some one of the common "balanced salt solutions" (Locke, Tyrode, Ringer, etc.) has usually been taken as the "negative control" and all experimental results compared therewith. It is known that chick cells will survive in Tyrode solution for 4 or 5 days, seldom more. It has seemed unnecessary to set up such a solution as a basis of comparison for every one of the nutrients tested in the course of this study, the mere fact of survival for periods of several weeks or months being

considered sufficient evidence of a nutrient's *qualitative* superiority to such a "control". In the same way the mass of literature on the unlimited survival of chick tissues *in vitro* in the embryo-juice blood-plasma media of Carrel and his successors constitutes a "positive control" which need not be repeated in every experiment. In this study, therefore, no controls in the usual sense have been introduced. The objectives as well as methods have, it is believed, been sufficiently different from those in common use to justify this omission for the present. When a nutrient of known composition capable of sustaining unlimited growth *in vitro* is available, that will then constitute a proper "positive control" for all subsequent work. Anything short of that appears to be of questionable value.

Each experimental series, however, has contained an arbitrary basis of comparison, a particular concentration of the ion or substance under investigation which is taken as "1". Concentrations of 0.1, 0.3, 1.0, 3.0 and 10.0 times this arbitrary standard have usually been studied in each series. Thus in studies on amino acids (see later) this initial control was taken as 15 mg. per liter, this being about the concentration found to be maximal for plant tissues. Concentrations of 1.5, 4.5, 15, 45 and 150 mg. per liter (total amino acid) were studied. When the experiments showed a regular increase in survival with increase in amino acid concentration it was clear that even 150 mg. per liter was not maximal and a second series was set up with 150 mg. as the control, and concentrations of 15, 45, 150, 450 and 1500 mg. per liter were tested. This again gave a regular increase in survival up to 450 mg. with a considerable decrease at 1500 mg.. A tentative optimum of 1000 mg. amino acid per liter solution was thus arrived at. The experiments have thus not been "without controls" although a single set of "positive" and "negative" controls uniform for all experiments has not been used.

Renewing Nutrient: One other matter of procedure requires consideration. If rapidly used nutrient constituents are not to be exhausted and toxic metabolic products are not to accumulate, the nutrient must be changed at frequent intervals. Rapidly growing skeletal muscle will drive the pH from 7.6 to 6.4 in three or four days. Heart muscle is less rapidly acidifying. Experience has shown that nutrient must be renewed at least every three or four days and in some cases it may be desirable to change oftener. This constituted one of the most serious difficulties at first, since many cultures were killed in

the process. A simple and effective procedure was finally developed.

For this purpose racks were provided as illustrated (Fig. 7). These had a base eight by eleven inches with a four-inch high upright set one and one-quarter inches from the back. The front was notched to take twelve tubes and notched stands were provided against the upright so that tubes could be laid at a 2° slope, or flat, or could be stood at an 88° slope against the upright. A strip of sterile filter paper (paper towel) was laid in the trough behind the upright and a second on the table in front of the rack. Twelve tubes were removed from the incubator and placed at the 2° slope. The tubes were then taken up one by one, and the stoppers swabbed off thoroughly with alcohol. The stoppers were removed and placed small end down on the front strip of filter paper, in order, the nutrient poured off and the tubes stood mouth downward behind the upright so that they drained onto the rear filter paper strip. After five minutes' draining they were recharged with fresh nutrient, the stoppers replaced and the tubes returned to the rack. Stoppers were again thoroughly washed off to remove any traces of nutrient—otherwise this will not only make the tubes sticky to handle but will serve as a breeding place for bacteria and molds—and were returned to the incubator. This procedure is effective and simple, and results in very few contaminations. Fifty tubes can be handled in this way in about a half-hour's time, an assistant being required only for the actual re-charging of the tubes.

Experimental Results

Using the nutrient described above as a standard and employing these methods, some very encouraging results have been obtained. They will be discussed under five headings.

Heart: Heart tissues from 5, 6, 7, 8, 9, 10, 12 and 14-day embryos were minced and streaked in tubes. Growth of both muscle and fibroblasts took place from all of these. From 5- to 8-day embryos showed a narrow band of fibroblasts within 24 hours (Fig. 8). With older embryos this might not become pronounced until 48 hours. In all cases the band of migrating fibroblasts was compact, the cells closely packed except at the margins and often so nearly isodiametric as to give an epithelioid appearance (Fig. 9). Subsequently the cells tended to elongate in the direction of migration. This band gradually widened and often attained a final width of 2 mm. or more. Muscle strands generally first became recognizable at about the third day,

although heart beat was evident in the explant within a few hours after excision. At first made up of single narrow strands, usually at the margins of cultures, these gradually extended until broad, strap-shaped bands and anastomosing nets were set up (Fig. 10). Contraction was at first confined to the original explant as was evident from the direction of pull, but after 6 to 8 days gradually became established in the outer bands until many cells were pulling away from the explant (Fig. 11). Contraction was generally strong and rhythmic. Adjacent strands might or might not contract synchronously.

With repeated renewal of nutrient the character of the tissue gradually changed. Fibroblasts tended to become more definitely spindle-shaped, the margins of the areas of migration often pulled away from the glass simulating the "liquifaction of plasma" so often illustrated in earlier publications. This apparent "liquifaction" occurred even when no plasma was present. These vacant areas were at first quickly closed by new zones of migration made up of spindle-shaped or triangular cells. Fatty degeneration began to set in after about two weeks (Fig. 12B). At first renewing the nutrient at 3 to 4-day intervals would revive these cells and the fat globules would be resorbed, but later degeneration became more pronounced. The fibroblasts rarely survived as definite well-developed areas for more than about two weeks (4 to 5 renewals of nutrient) although isolated cells continued to migrate away from the explant for much longer periods.

While the fibroblasts underwent progressive degeneration after the first ten days, the reverse was true of the muscle bands. These generally reached their maximum development after two to three weeks, and thereafter showed little regression or degeneration not directly traceable to injury of some sort. One culture showed beautiful rhythmic contraction up to the 28th day when it became contaminated with a mold. Another continued to beat for 44 days through 5 renewals of nutrient. Unlimited survival has not yet been obtained but the results are such as to suggest failures of technique rather than nutritional failure as the causes of death.

Skeletal muscle is much more moist when excised than heart tissue and does not initially adhere to the glass nearly so well. The original definite linear arrangement of explants is seldom recognizable after 24 hours, as it is with heart muscle. Nevertheless, all fragments gen-

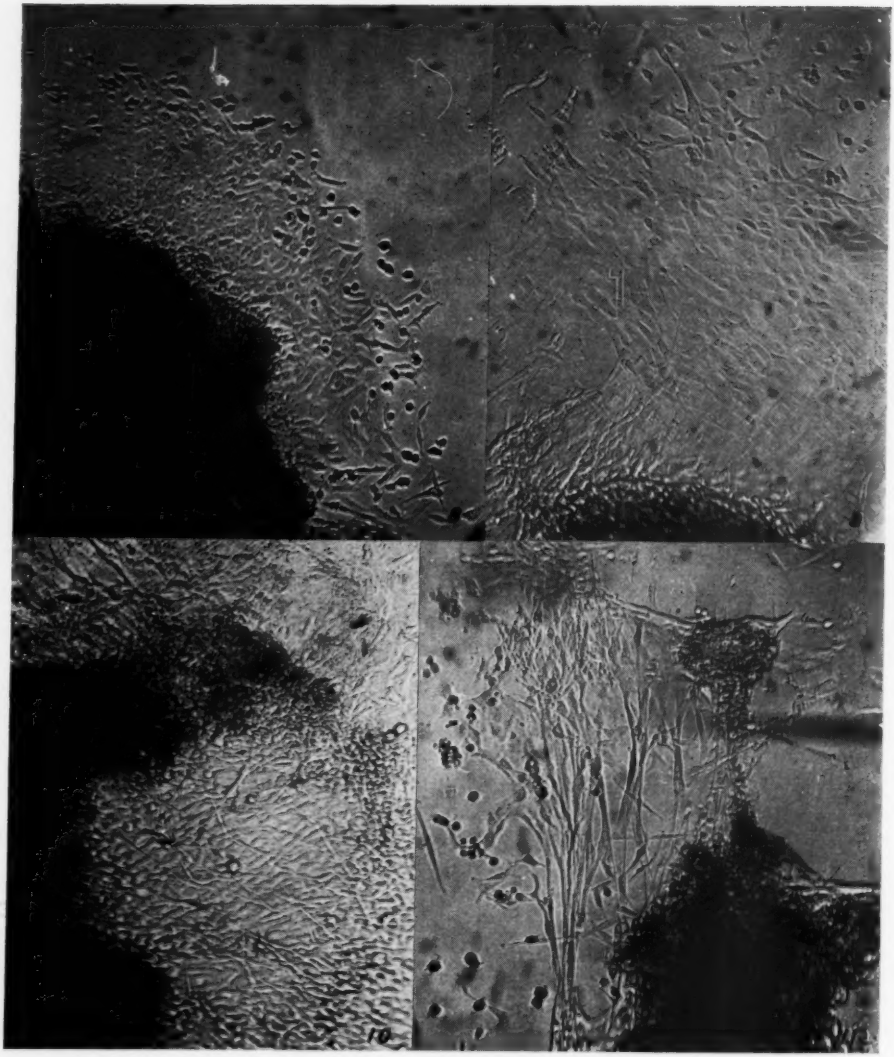

FIGURE 8
Fibroblasts from heart tissue showing the usual pattern. At 24 hours. (X 90)

FIGURE 9
Epithelioid fibroblasts from heart tissue. At 4 days. (X 90)

FIGURE 10
Heart muscle and skeletal muscle in mixed culture. 17 days; 1 renewal of nutrient. Heart muscle in beautiful widespread pulsation. (X 90)

FIGURE 11
Heart muscle. 12 days; nutrient renewed once. Actively pulsating. (X 90)

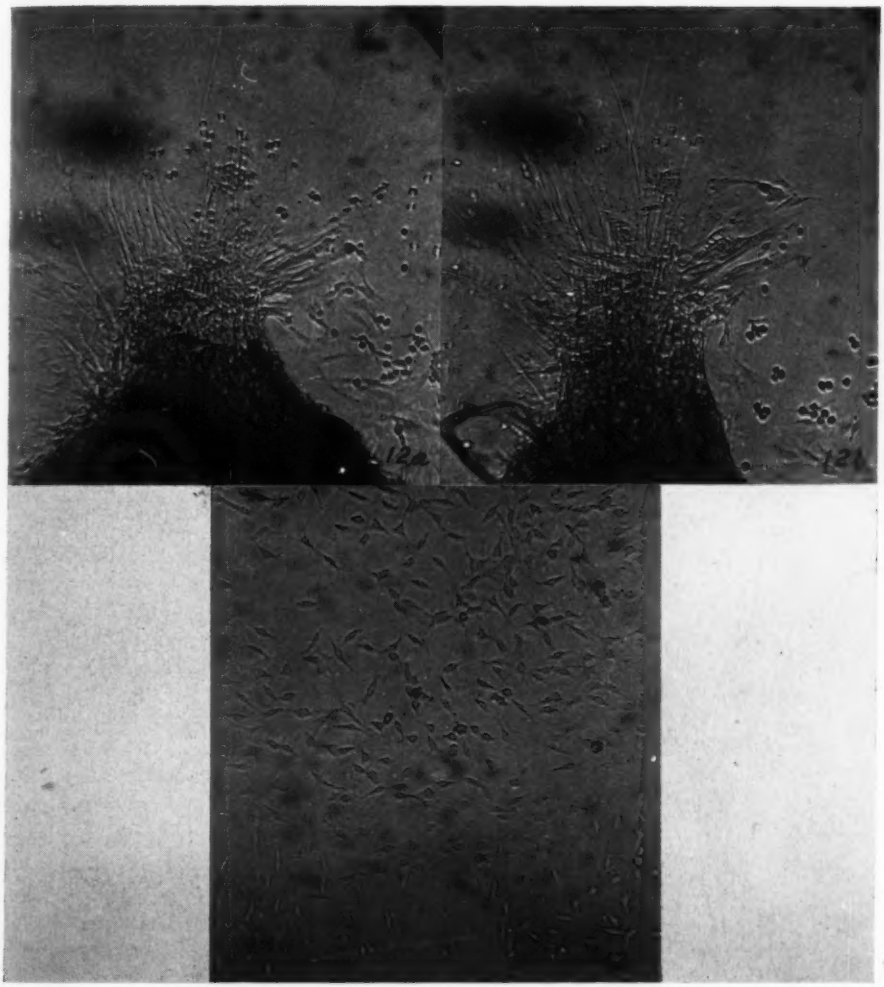

FIGURE 12A

Heart muscle. 12 days; nutrient renewed once (after 11 days). Actively contracting. (X 84)

FIGURE 12B

Same as Figure 12A; 72 hours later. Contracting. Note especially extension of fibers at top, center, reorientation and beginning vacuolar degeneration at top right. (X 84)

FIGURE 13

Fibroblasts from skeletal elements. 4 days. Photograph taken near margin of culture, about 5 mm. from explant, showing loose, open migration of characteristically spindle-shaped cells. (X 84)

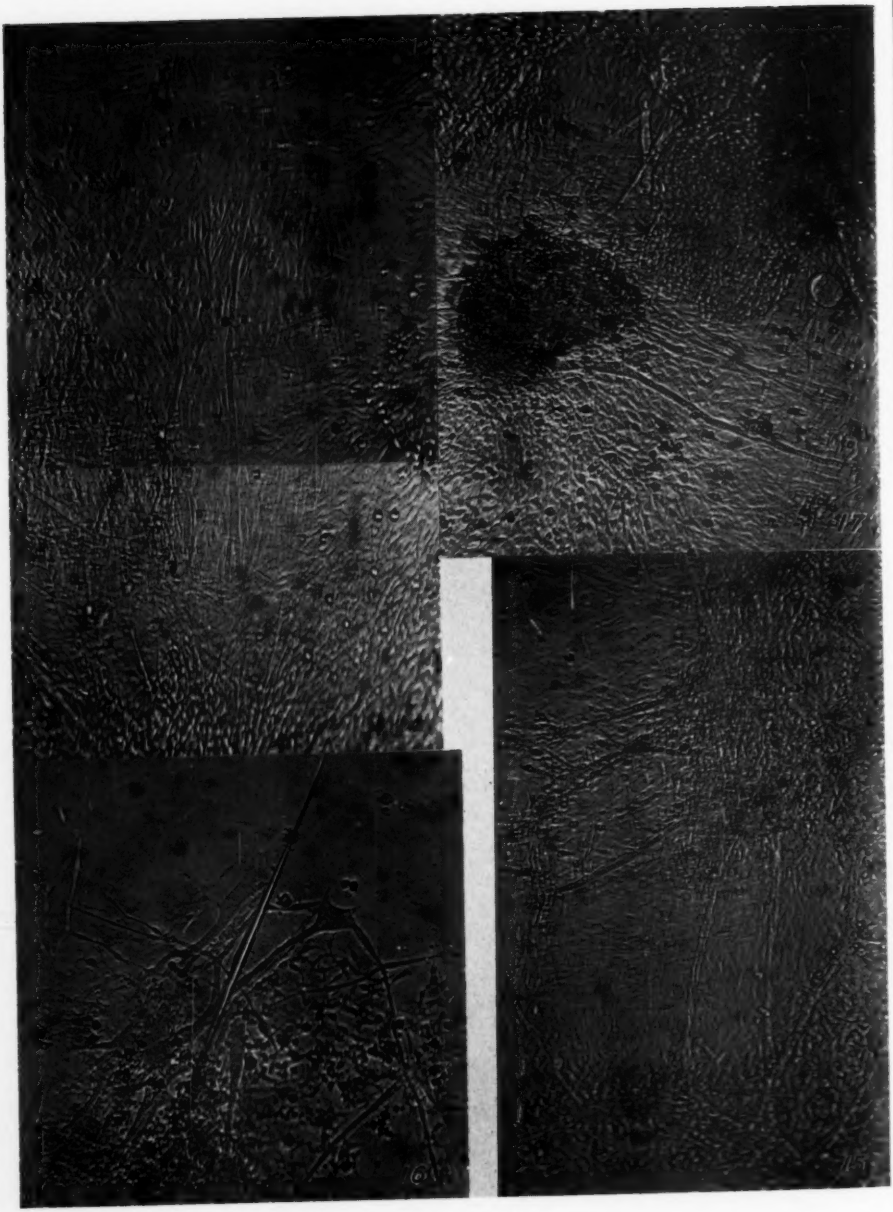

FIGURE 14

Skeletal muscle. 7 days. Note the strap-shaped muscles which overlie the fibroblast sheet. (X 86)

FIGURE 15

Skeletal muscle. 7 days. Here the muscle strands form an anastomosing net. These strands were probably already present and have been stretched out by the migrating fibroblasts, while those in Figure 14 appear to be newly formed. (X 86)

FIGURE 16

Skeletal muscle, at 3 days, showing the typical broad bands formed when these cell groups migrate beyond the fibroblast margin. The spindle-shaped object is a flaw in the glass of the culture tube. (X 86)

FIGURE 17

Skeletal muscle, at 7 days, showing muscle bands overlying fibroblasts. The round object (right, center) is a muscle band which has torn loose and contracted. Some cells at the top, center which are highly refractive (rounded) are beginning to contract. (X 86)

erally do attach themselves, though tardily and at random, so that none are floating free after 48 hours. The band of migration is much more quickly established, the character of growth looser, with less tendency to isodiametric, epithelioid patterns. The cells are smaller, more definitely spindle-shaped, and may lie in one or several layers. The massive migratory sheet may extend as much as 5 or 6 mm. from the explant and single cells may be found scattered over the entire surface of the glass (Fig. 13). This sheet is compact but the cells are oriented in rather definite patterns, apparently due to tensile strain-lines on the one hand, and definite skeletal elements on the other. These skeletal elements are in the form of muscle strands which are for the most part longer and narrower than heart muscle (Fig. 14) and differ also in becoming evident within 24 hours after implantation. They reach their greatest development within the first four to five days, and then rapidly disappear. There is, in fact, the suspicion that they may sometimes represent strands and networks already in the explant at the time of excision (Fig. 15), and drawn out of the explant by the migrating mass of fibroblasts rather than growing out of their own volition. This is clearly not always true, however, for single strands can often be seen pushed far out beyond the margin of the fibroblast sheet (Fig. 16). This is especially evident on a plasma substratum. Moreover, this muscle-net, which disappears while the fibroblast sheet is in its prime, often reappears in very old cultures where the fibroblasts are degenerating. These strands may be arranged as a radiating "wheel" with the explant as a center or they may be anastomosed into a regular honeycomb net, or they may be completely random in arrangement. They are at first narrow but after six to eight days begin to broaden. The tips often expand into ovoidal plates, the muscles contract longitudinally forming spherical structures representing a median phase of degeneration (Fig. 17). These finally disappear completely, leaving only the fibroblast sheet.

One striking feature of these skeletal muscle strands is their autonomous pulsation, a phenomenon noted by Pogogeff and Murray (69). This is infrequent but not rare. Long strands are seen to contract vigorously in a rhythmic fashion. The pulsation is usually very powerful, the initial response being abrupt followed by a gradual relaxation. The rhythm under such conditions is usually slow, once in 5 to 8 seconds, and seldom continues for long periods unbroken.

In this it differs from the characteristic heart-muscle pulsation. Sometimes a strand will contract at first with a slow pulse but with a rhythm which accelerates rapidly to a fibrillating tremor, after which the strand will relax completely and remain quiescent for several seconds. The same progressively accelerating contractile phase will then be resumed. It would be extremely interesting to know the stimulatory mechanism responsible for these contractions.

With the initial disappearance of the muscle bed, which is usually complete after about eight to ten days, the fibroblast sheet continues in good condition for many weeks (Figs. 18-22). There is a gradual piling up of fibroblast masses at points distant from the original explant (Fig. 18), which have the appearance of new centers of growth and from which it may ultimately be possible to make subcultures. Such sheets have seldom survived for more than fifty days. Fatty degeneration ultimately sets in, followed by a rounding up of the fibroblasts and a shrinkage of the sheet, with final disintegration and death (Figs. 21, 22).

Excellent cultures for general study can be prepared by mixing heart and skeletal elements before mincing. Cultures so prepared have the regular adhesive qualities of heart muscle combined with the broad fibroblast migration areas of skeletal muscle. The heart muscle strands tend to extend to somewhat greater lengths on a skeletal fibroblast substratum than when they have to lay down their own foundation. They do not, however, survive any longer nor is the optimum extension any greater than with the best pure heart muscle cultures.

Epithelium: Epithelium is occasionally found in cultures taken from any general area of the body and beautiful cultures have been obtained from heart (pericardium?) (Fig. 23), brain, liver (Fig. 24), and skeletal muscle as well as skin. All are characteristic, with relatively isodiametric or rhomboidal, never spindle-shaped cells and sharp sheet margins. The most consistently good epithelial cultures have been obtained from the amniotic membrane. Epithelial growth of such cultures takes two forms. First there is the flat type in which growth results in a single layer of cells closely appressed to the glass. This is the only type observed with pericardium, liver, skeletal or brain elements. The sheet spreads quite rapidly and at the end of six to eight days may extend as much as 5 mm. from the explant. Sheets from adjacent explants frequently fuse (Fig. 25) so that broad mem-

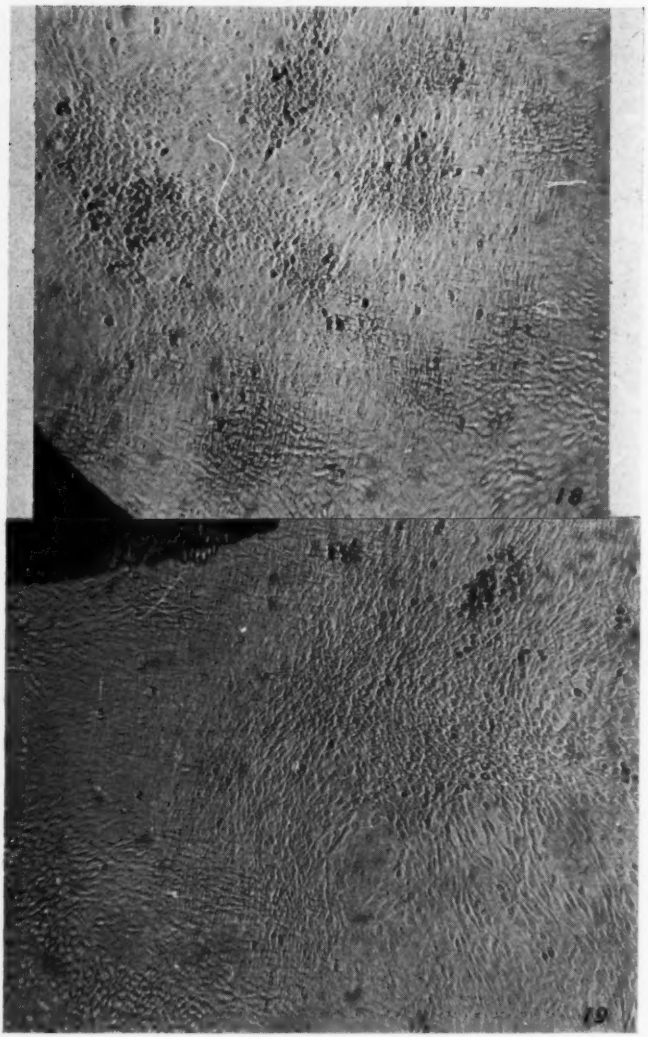

FIGURE 18

Skeletal fibroblasts, at 40 days; nutrient renewed 5 times. This is the same culture as that shown in Figures 19, 21 and 22. (X 100)

FIGURE 19

Skeletal fibroblasts, at 30 days; nutrient renewed 3 times. Compare with Figure 21 (same culture). (X 100)

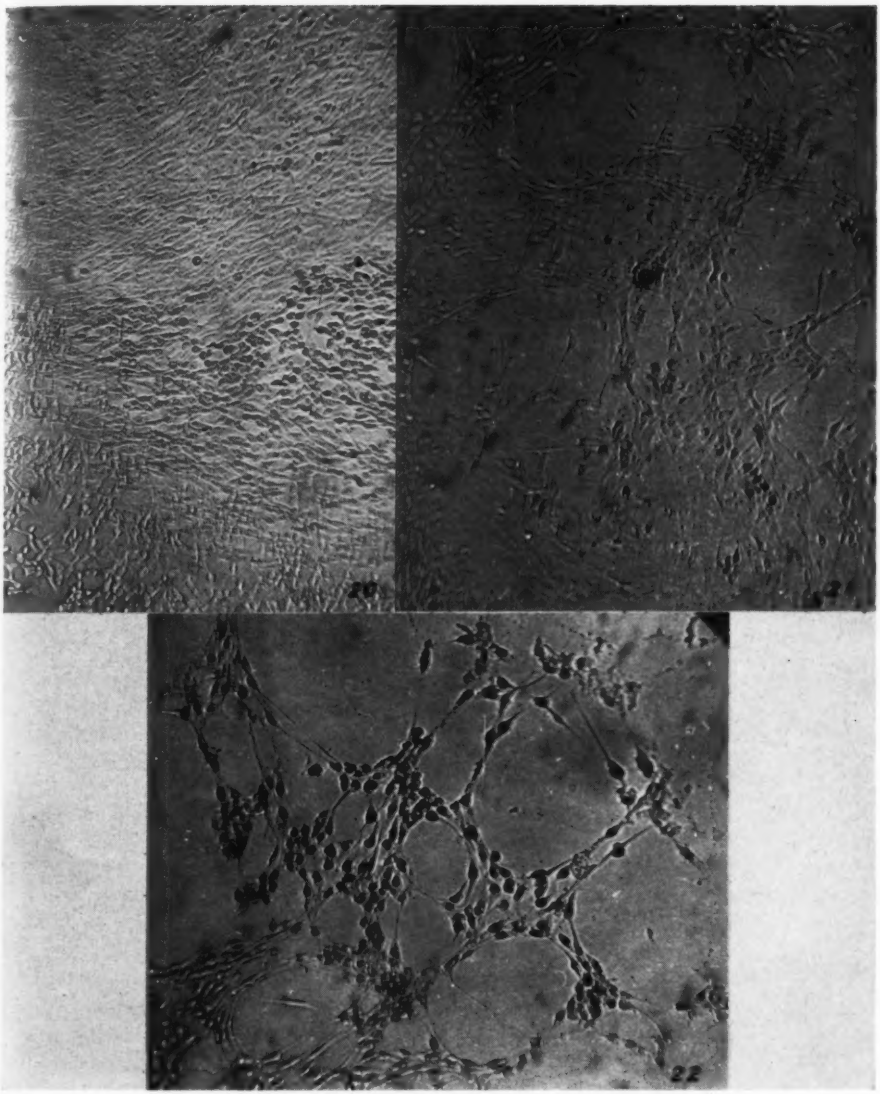

FIGURE 20

Skeletal fibroblasts, at 6 days; nutrient renewed once. Note the difference between the upper half in which the cells are in excellent condition and the lower half where the cells have rounded up and begun to degenerate. These came from two separate explants, probably from different eggs, and show the great differences in survival value of different embryos under identical culture conditions. (X 94)

FIGURE 21

Skeletal fibroblasts, at 42 days; nutrient renewed 5 times; beginning to degenerate. (X 94)

FIGURE 22

Skeletal fibroblasts, at 40 days, and after 5 renewals of nutrient; showing marked degeneration. (X 94)

branes extending over considerable areas of the culture tube may be formed. Where the margin of a sheet is moving forward it is so thin as to be all but invisible. Only where it has begun to retract and roll up (Fig. 25) does the margin become clearly defined. This contraction, which begins after about ten days, results in tearing of the

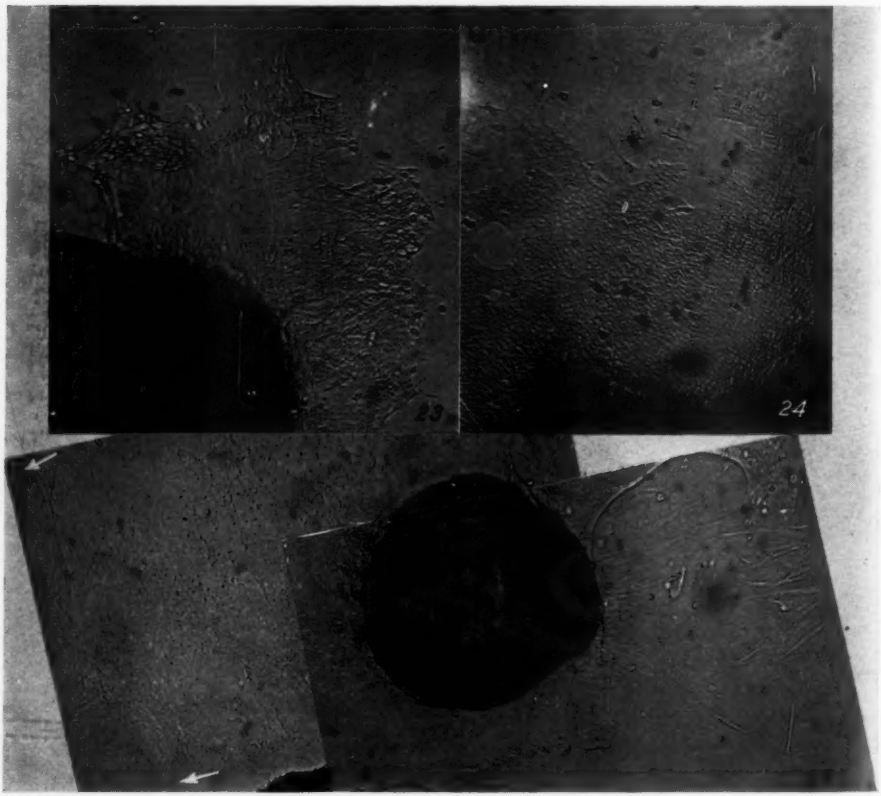

FIGURE 23
Pericardial epithelium, at 3 days. (X 78)

FIGURE 24
Liver epithelium, at 3 days. (X 78)

FIGURE 25
Amniotic epithelium, at 9 days; nutrient was renewed once. Note the line of suture at the extreme left where epithlium from two separate explants has fused to form a continuous sheet. Retracted (rolled) margin and overlying fibroblasts shown at the right. (X 78)

membrane. Often a layer of fibroblasts has already been laid down under this membrane and becomes evident through the tears (Fig. 26). Fibroblasts also sometimes migrate beyond the epithelial margin after it has ceased to extend. Epithelium seldom survives in good condition for more than two weeks although the fibroblasts may survive for a much longer time.

In addition to this flat type of growth, epithelium from amnion and from feather follicles may develop into vesicles which are strikingly characteristic. From feather follicles these are usually one- or few-celled. From amnion they may vary all the way from a few cells in size up to organules several millimeters in diameter (Fig. 27). The larger types are traversed with trabecular strands (Fig. 28). From about the third to the sixth day these strands are in constant slow motion resembling that of the protoplasmic strands within a stamen hair or those observed in cultured ovarian cells of *Antirrhinum* (White, 96). This trabecular movement may be quite random, especially in small vesicles but in larger vesicles it sometimes becomes synchronous resulting in slow, powerful, peristaltic movements. The rhythm of this movement has a cycle of seldom less than 3 or 4 seconds, and may also be intermittent. It is an extraordinary thing to watch.

These vesicles may be the center of a flat membrane of the first type, or they may be attached to the glass without lateral extension, or they may often be found floating free in the nutrient (Fig. 27). When thus free they bear a striking resemblance to tiny specimens of the brown alga *Vâlonia*. Peristaltic movement in these vesicles is relatively short-lived but the vesicles themselves, while contracting and sometimes flattening out, do survive for several weeks.

Nerves: Nerve fibers are of special interest. Occasional nerve processes have been found in all types of culture except amnion and some of the best single examples are from heart (Fig. 29). The best source of nerve material has been found to be the dorso-caudal region of the body. This contains spinal ganglia which give rise to a profuse growth of fibers. These always arise, no matter from what organ —heart, liver, skeletal muscle, brain or spinal ganglia—from a characteristic very finely-granular mass without recognizable cell boundaries and with which fibroblasts are never intimately associated except secondarily (by invasion). The fibers themselves are extremely tenuous, for the most part perfectly straight except at the tips, and occur

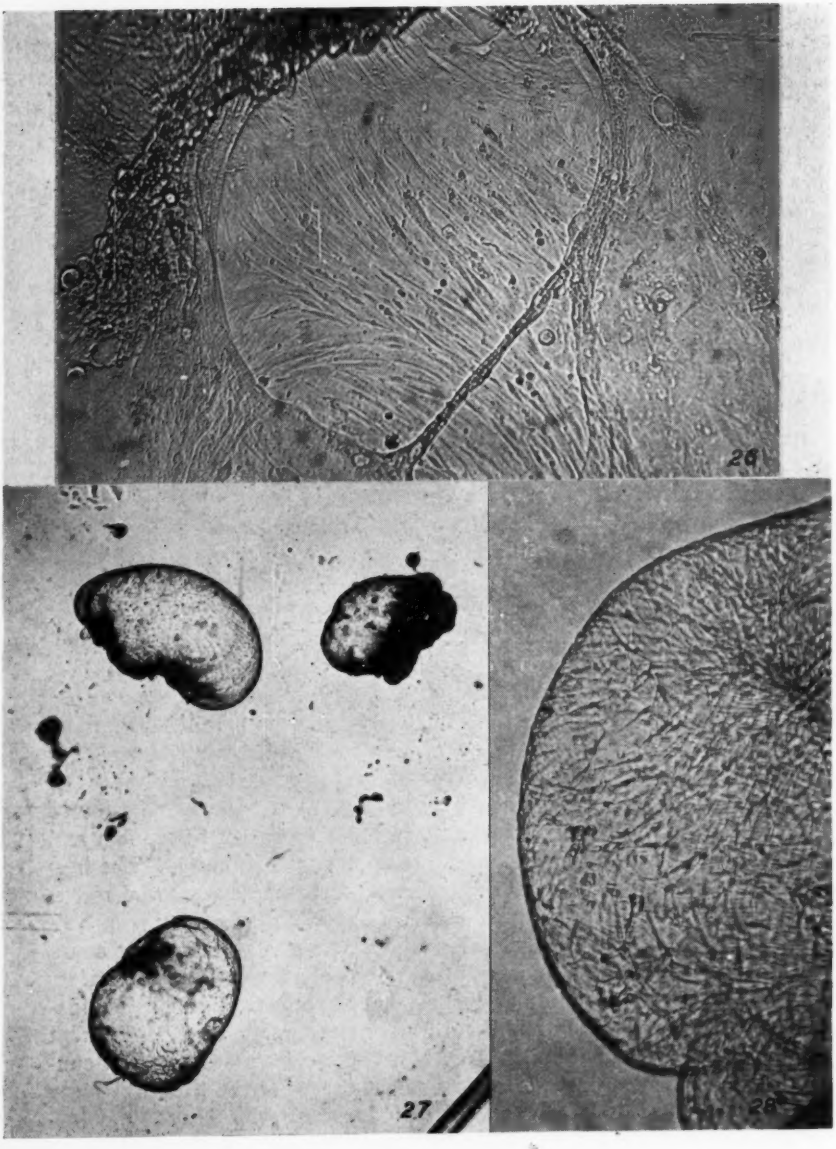
26
27
28

FIGURE 26

Amniotic epithelium, at 9 days; nutrient was renewed once. The membrane has torn, showing the overlying layer of fibroblasts. (X 100)

FIGURE 27

Epithelial vesicles floating free in the nutrient, at 4 days. These often show pronounced peristaltic movements. (X 13.5) (Photograph by J. A. Carlile.)

FIGURE 28

Detail of vesicle similar to those shown in Figure 27, showing the slender trabeculae which traverse these structures in all directions and which are in constant motion. (X 100)

either singly (Fig. 30) or in cables (Fig. 31), or occasionally in broad bands. Often a nerve fiber will run first to one cell, then with an abrupt change of direction, to a second, then on to a third in a new direction with occasional forks (Fig. 29). The tips are very slender and may be branched (dendrites) or attached to single cells (end-organs, fibroblasts?). The nerve fibers have been observed to grow outward from the original explant, to change position from day to day, and finally to disappear. Neural elements apparently do not survive for more than about two weeks under the conditions used.

General Considerations

This paper has described a technique, based upon that of Gey and Gey but with many modifications, for the cultivation of animal cells *in vitro* in a nutrient of fully known and exactly duplicable composition. With this method four major types of cells of the chick embryo —heart muscle (and fibroblast), skeletal connective tissue and muscle, epithelium with its accompanying fibroblasts, and nerve, have been maintained alive and in varying degrees of activity for a maximum of 56 days, through a maximum of 9 renewals of nutrient. The de-development and subsequent degeneration of such cultures has been followed in some detail.

All these tissues have, it is true, been maintained by other workers *in vitro* in complex nutrients of unknown composition for much longer periods. It is believed, however, that none has been maintained with anything like this degree of functional effectiveness for comparable periods, in nutrients *of known composition* as has been the case here. It is believed, therefore, that although falling short of the goal of *unlimited* growth, these cultures do represent an advance over previous work and indicate that the goal originally set is by no means a hopeless one.

What has been said so far was organized with the intention of first defining the problem, second presenting the technique in as simple terms as possible so that it may be available to other students, and third indicating in a general and admittedly preliminary way, the sort of results which can already be obtained with this technique. Discussion of the particulars by which this technique was developed has been avoided purposely, since such discussion would only distract from the reader's grasp of the procedures themselves. Such a discussion is, however, pertinent and will be presented at this point.

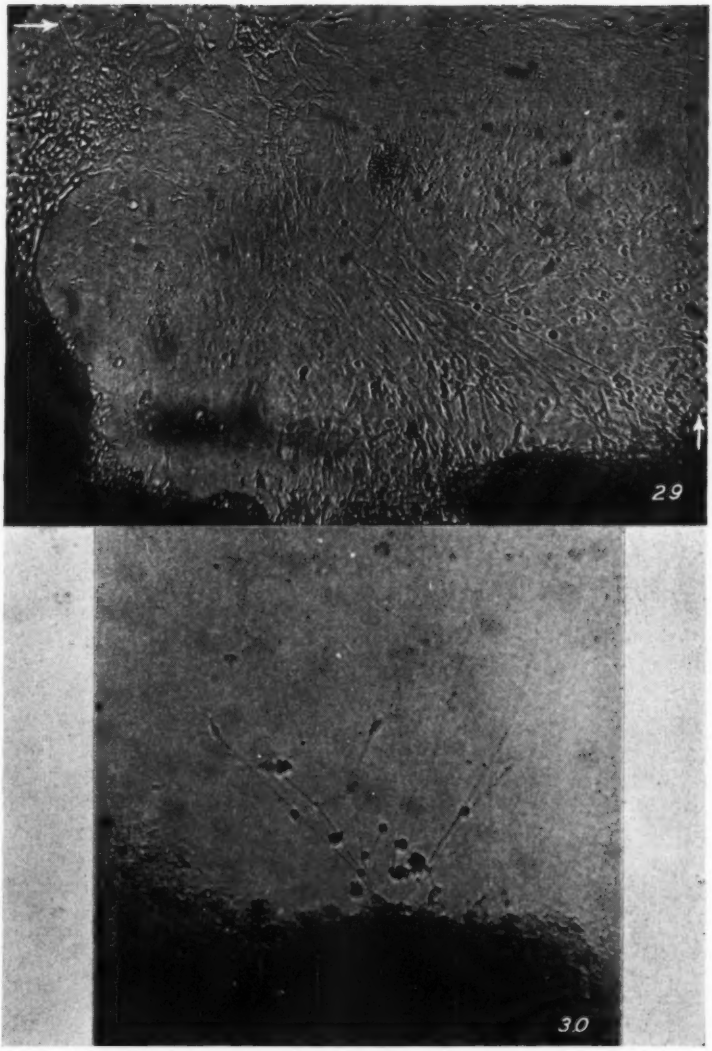

FIGURE 29

Skeletal muscle and heart muscle mixed, at 24 hours. Photograph shows a nerve fiber (see arrows) running from the lower right-hand to the upper left-hand corner. Compare the dimensions of the nerve fiber with those of the underlying fibroblasts. (X 100)

FIGURE 30

Spinal ganglion, at 24 hours, showing early stage of nerve fiber development. (X 100)

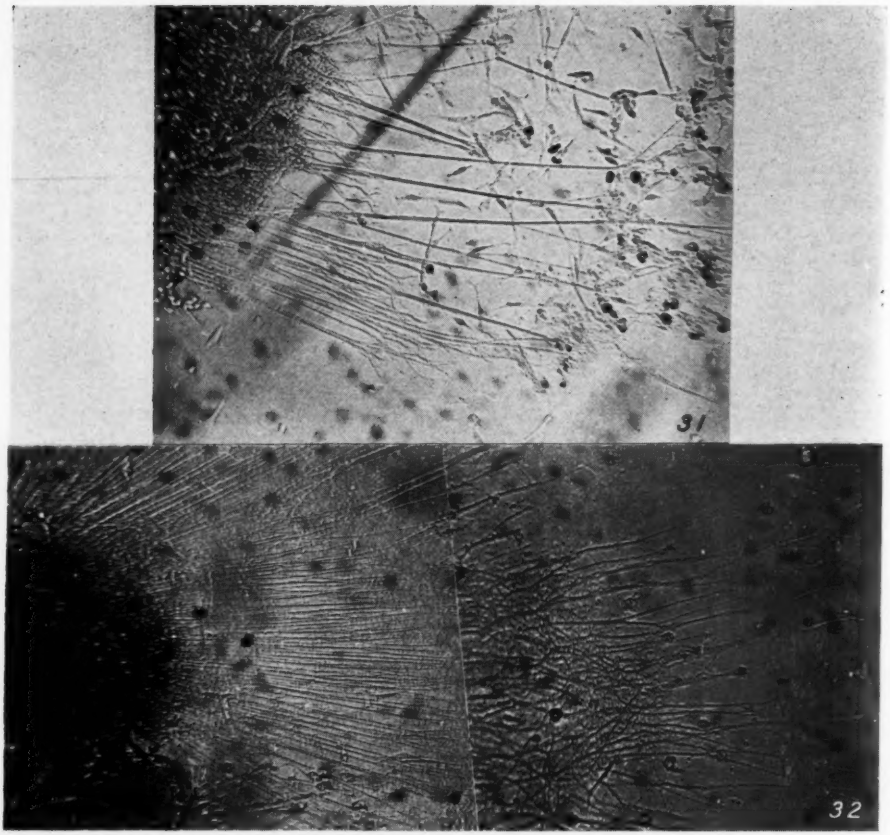

FIGURE 31
Spinal ganglion, at 7 days. Compare with Figure 30. (X 84)

FIGURE 32
Spinal ganglion, at 7 days. Note the plexus in the right-hand part of the picture (tangled fibers) with straight fibers to right and left. (X 84)

The nutritional problems involved divide themselves naturally into four groups, the studies of which are characteristic of the method of approach in the preliminary stages of this investigation. These are: energy sources, inorganic salts, organic nitrogenous building blocks,

and organic catalysts. None of these has yet been studied in anything like adequate detail. Nevertheless, it seems desirable to present what has been done as a representation of method rather than with any thought of giving a complete or rounded picture.

Energy Sources: Tissues of any sort, either plant or animal, obviously cannot be expected to continue to develop or even to maintain themselves without some source of respiratory energy. In most animal tissue culture studies this requirement has been met by the unknown sorts and amounts of carbohydrates and other combustible materials present in plasma and embryo juice. These are sometimes supplemented with small amounts of dextrose. Gey's formula, for example, provides 0.1 per cent. dextrose (42, 43). Compared to the 2 to 6 per cent. of sugar which is known to be optimal for plant tissues, this is clearly very low (114) (Bonner and Addicott, 19).

Dextrose is the only sugar that has commonly been added to animal tissue nutrients. Most of the rather extensive work with plant tissues, on the other hand, has shown the disaccharides and polysaccharides, especially sucrose, to be utilized in more adequate fashion than are the monosaccharides (White, 100, 108, 109; Thielmann, 87; van Overbeek, 65; Bonner and Addicott, 19; and others). A theoretical basis for this apparently anomalous observation is to be found in the work of Doudoroff, et al., (34), Hanes (46), and others, who have shown that in the respiratory transformations the energy requirement for phosphorylation at the expense of inorganic phosphate to produce the respiratory intermediate dextrose-monophosphate is met more effectively by the disaccharides, sucrose and maltose, than by dextrose, one hexose moiety furnishing the energy for phosphorylation of the other. Besides this theoretical superiority as a respiratory substrate sucrose has the great practical advantage of being stable to autoclaving, which dextrose is commonly thought not to be, and of being available in a much purer form than is any of the monosaccharides. It seemed desirable, therefore, to determine first whether or not sucrose could be substituted for dextrose in such formulae as that of Gey, and second, whether the concentration of carbohydrate could be increased above the 0.1 per cent usually recommended.

This second objective offers certain practical difficulties. In plant tissue nutrients the carbohydrate has a double rôle, as energy source and also as the major osmotic agent. These nutrients do not contain sodium chloride. In animal tissue nutrients, on the other hand, the

TABLE 1

EFFECTS OF CARBOHYDRATE: NaCl RATIOS ON THE BEHAVIOR OF CHICK EMBRYO TISSUES IN TISSUE CULTURE

	Normality ratios (Total value = 0.2815N)	Wt. % of sugar	Wt. % of NaCl	Survival, increment and activity after, days											
				1			2			3			4		
				Growth	Heart beat	General condition	Growth	Heart beat	General condition	Growth	Heart beat	General condition	Growth	Heart beat	General condition
Dextrose	1:12	0.45	0.76	+	+	+	+	+	+	+	+	+			
				+	+	+		+							
	1:6	0.85	0.7	+	+	+	+	+	+	+	+	+	+	+	+
				+	+	+	+	+	+	+	+	+	+	+	
										+			+		
	1:3	1.5	0.6	+	+	+	+	+	+	+	+	+			
					+										
	1:1	3.0	0.4	+	+	+	+	+	+	+	−	+			
				+	+		+	+							
Sucrose	1:12	0.9	0.76	+	+	+	+	+	+	+	+	+	+	+	+
				+	+	+	+	+	+	+	+	+	+	+	+
					+		+	+	+	+			+		
	1:6	1.7	0.7	+	+	+	+	+	+	+	+	+	+	+	+
				+	+	+	+	+	+	+	+	+	+	+	+
							+	+	+	+	+		+	+	
	1:3	3.0	0.6	+	+	+	+	+	+	+	+	+	+	+	+
				+	+		+	+							
	1:1	6.0	0.4	+	+	+	+	+	+	+	+	+	+	+	+

major osmotic rôle is played by NaCl. To increase the sugar concentration without corresponding reduction in NaCl would disturb the isotonicity of the solution. This may or may not be important. To reduce the NaCl concentration in order to maintain isotonicity might, on the other hand, upset the balance between the cations Na:K:Mg which is likewise supposed to be important. Gey's solution has a total osmotic value of about 8.4 atm., about five times that of optimal plant tissue nutrients. This is made up as follows: NaCl, 7.04 atm., dextrose, 0.14 atm., salts other than NaCl, 1.21 atm. The ratio of dextrose to NaCl, on a normality basis is 1:50, on a weight basis 1:8. Isotonicity can be maintained in experimental nutrients by varying this carbohydrate:NaCl ratio at a constant sugar-plus-

TABLE 1 (*continued*)

Survival, increment and activity after, days																				
5			7			9			11			13			15					
Growth	Heart beat	General condition	Growth	Heart beat	General condition	Growth	Heart beat	General condition	Growth	Heart beat	General condition	Growth	Heart beat	General condition	Growth	Heart beat	General condition			
+	+	+	+	+	+															
+	+	+																		
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
+	+		+	+			+			+		+	+	+		+				
+	+	+	+	+	+	+	−	+	+	−	+	+	+	+	+	+	+			
+	+		+										+	+		+	+			
+													+			+				
+	+	+	+	+	+	+	−	+	+	−	+									

NaCl value of 7.18 atm. or 281.5 mN. Preliminary experiments showed that the dextrose concentration could be increased at least tenfold without seriously retarding growth of cultures.

To test the relative availability of dextrose and sucrose and to elaborate on the question of optimum carbohydrate concentrations, a series of eight solutions with carbohydrate:NaCl ratios varied between 1:12 and 1:1 (molar basis) at constant normality was prepared. Five tubes, each containing 10 to 20 cultures of mixed heart and skeletal muscle of 7-day chick embryos were set up in each solution. Fibroblast migration, heart beat and general appearance were recorded over a period of three weeks, without renewing the nutrient.

The experiments were repeated three times. Results of a typical experiment are shown in Table 1.

The results are reasonably clear-cut. With dextrose the ratio carbohydrate:NaCl (molar basis) of 1:6 is definitely optimal, heart beat continuing for 7 days at this ratio, for only 3 days at 1:12 and 1:3, and for only 2 days at 1:1. This is a weight concentration of 0.85 per cent., eight and one-half times that recommended by Gey (42). With sucrose the same ratio is optimal, heart beat being maintained for 22 days (!) at 1:6, 15 days at 1:12, 7 days at 1:3 and only 4 days at 1:1. This is a weight concentration of 1.7 per cent.. It is interesting to compare this with the 2 per cent. sucrose considered optimal for tomato roots (White, 114; Bonner and Addicott, 19; and others).

This does not, however, tell the whole story. Fibroblast migration is very much more active during the first few days in dextrose than in sucrose. With skeletal muscle explants there is also a much more rapid fall in pH in dextrose than in sucrose. This is interpreted as indicating a more rapid metabolism (utilization). It seems probable that dextrose is superior to sucrose if the nutrient is to be changed at frequent intervals, while sucrose is superior if nutrient is not to be frequently renewed. This work contemplates frequent changes of nutrient as a means of demonstrating the real adequacy of nutrition. Dextrose has therefore been used. Sucrose, however, deserves a much more thorough examination. The behavior seems somewhat analogous to that of tomato roots *vis à vis* iron concentrations, where a concentration of 0.2 mg. per liter appeared optimal over short term experiments (1 week), but in experiments involving six renewals of nutrient this proved quite inadequate, a concentration of 0.6 mg. per liter being optimal (White, 102).

Inorganic Salts: The inorganic constituents of the nutrient immediately presented a number of serious problems. The standard inorganic diluents in common use are all modifications of Ringer's, Tyrode's or Locke's solutions. A glance at their formulae shows their inadequacy from any considered theoretical point of view. Ringer's solution consists of NaCl, KCl and $CaCl_2$ (70). Tyrode adds to these $MgCl_2$, NaH_2PO_4 and $NaHCO_3$ (88). Locke adds KH_2PO_4 (57). Drew substitutes $CaH_4(PO_4)_2$ and $MgHPO_4$ for the NaH_2PO_4 (35). All of them are completely lacking in: *1*) sources of the inorganic nitrogen or sulfur which are so important to plant tissues, and *2*)

sources of iron, copper, zinc, manganese, iodine and fluorine, all of which are known to be essential for the proper functioning of at least some animal tissue systems. These must be supplied either in inorganic or organic form. The assumption (not, so far as I can discover, based on any evidence) is generally made that they will be supplied in unknown but adequate amounts in the organic supplements, tissue broths, etc., which are generally used. This is one of the assumptions which the present work is intended to eliminate.

Gey's solution (a modified Locke solution) was chosen as a basis for preliminary study. It was originally developed so as to approximate the constitution of whole blood as regards the major ions. For comparison the concentrations in whole blood, as given by Hawk and Bergeim, are listed below.

Element	mM	
	Whole blood	Gey's solution
Na	143	138
Ca	2.5	2.3
K	5	5
Mg	0.8	1
Cl	140	146
SO_4	0.1	—
PO_4	0.4	0.5
HCO_3*	25*	3.3

*Figures from Starling (84); not given by Hawk and Bergeim (48).

This solution is constituted as follows: NaCl, 8.0 g.; KCl, 0.375 g.; $CaCl_2$, 0.250 g.; $MgCl_2.6H_2O$, 0.210 g.; $Na_2HPO_4.12H_2O$, 0.120 g.; KH_2PO_4, 0.026 g.; $NaHCO_3$, 0.275 g.; H_2O, 1000 ml..

In altering the ratio of dextrose to NaCl from 1:50 (Gey's formula) to 1:6 (weight per cent. of dextrose increased from 0.1 per cent. to 0.85 per cent.) it was necessary to reduce the NaCl concentration from 138 to 121 mM. Preliminary experiments also showed that equimolecular concentrations of $Ca(NO_3)_2$ and $MgSO_4.7H_2O$, which are standard in plant tissue culture nutrients, could be substituted for the $CaCl_2$ and $MgCl_2.6H_2O$, respectively, without affecting the growth of cultures adversely. These changes introduced the NO_3 and SO_4 radicals, the desirability of which has already been suggested. The NO_3 concentration of whole blood is not recorded in Hawk and Bergeim although some of the "non-protein N" is doubtless in this form. Equimolecular substitution of the sulfate for the chloride of magnesium gives 1 mM of SO_4, which is ten times the concentration in

whole blood. These substitutions did not result in clearly *improved* growth, but were made for theoretical reasons.

Experiments were then undertaken to determine optimal concentrations for each of these ions. Results recorded were obtained with cultures of mixed heart and skeletal muscle from 8-day chick embryos in which maintenance of heart beat and migration and survival of fibroblasts in cultures incubated without renewal of nutrient were used as criteria. They are, therefore, to be considered tentative and will require verification using repeated passages after optimal levels for other culture conditions have also been established. As a result of these experiments the concentration of calcium was reduced from 2.3 to 1.3 mM with a consequent establishment of NO_3 at a 1.3 mM level, the concentration of K remained unchanged, that of Mg was reduced from 1 to 0.6 mM with the establishment of the SO_4 concentration at 0.6 mM, (still considerably higher than the 0.1 mM of whole blood), and the bicarbonate concentration was increased from 3.3 to 6.6 mM. This last concentration is still only one quarter that of whole blood. It was also found possible to introduce iron as $Fe(NO_3)_3.9H_2O$ at a concentration of 1.75 mM. This is only one third of the iron concentration of whole blood and will obviously require further study. The nitrate was used rather than some other salt because it makes a stable stock solution which does not precipitate out as does the sulfate and is less deliquescent than the chloride. The citrate or tartrate are, of course, to be avoided because of the potential (and unknown) nutritive value of the organic radicals.

With a "first approximation" established for the major ions the question of "trace elements" was also examined in a preliminary way. Hawk and Bergeim (48) give the concentration in whole blood of iron as 5.2 mM, copper as 0.08 mM and iodine as 0.0012 mM. No other trace ions are considered. It seems obvious that, in view of the well recognized concentration of iodine in the thyroid, its function as part of the thyroxine molecule and its therapeutic rôle in treatment of pathological conditions resulting from thyroid disfunction, one should not rationally expect to study the physiology of tissue cultures of thyroid in a nutrient devoid of iodine. The concentration of zinc in the pancreas and its use in preparation of crystalline insulin creates a comparable picture as regards cultures of the Islands of Langerhans. The concentration of copper in the blood and perhaps the liver and the concentration of iron in these same tissues indicate the probable

importance of these ions. It is well known that iron, zinc, manganese and boron are essential for proper function of most plants, and copper, iodine and molybdenum for at least a few (Arnon, and others, 1-4, 85). Experiments with chick embryo tissues were carried out with iron, zinc, copper, manganese, cobalt, molybdenum, iodine, fluorine, bromine, silicon and boron, singly and in various combinations. While all were tolerated singly, many at concentrations which seem extraordinarily high to a plant physiologist, any considerable group of these ions proved depressant and there was no clear-cut evidence of their being essential. While these results can be only tentative and it seems almost certain that in long term experiments, once a more satisfactory technique has been established, some of these may well prove to be essential, it has seemed best to omit them for the present.

The use of crystalline zinc-insulin and of thyroxine (see under vitamins) likewise proved of no clear-cut benefit and was abandoned. The citrates, malates and pyruvates have not yet been examined.

Organic Nitrogenous Building-Blocks: It is well known that all of the organic nitrogenous requirements of rats and dogs, and probably of other animals as well, can be met by a mixture of a comparatively small number of amino acids (Rose, 79) with the addition of some nitrogen-containing organic catalysts (?) such as hormones and vitamins. It is also known that a similar mixture of amino acids can be substituted for the serum proteins in the treatment of some forms of shock, and that they are quickly assimilated into the working system (Madden and Whipple, 60; Madden, *et al.*, 61). Except for some early work by Burrows (21) and the abortive studies of Baker and Carrel (8), little appears to have been done with the amino acid nutrition of animal tissue cultures and nothing whatever which is in any way based on our contemporary knowledge.*

With these facts in mind studies were carried out using Madden and Whipple's formula for amino acids, without change. Experiments

*Since completion of this manuscript there has come to my attention a paper by Fischer published in Sweden in 1941 but only now available because of the war (Fischer, A., Die Bedeutung der Aminosäuren für die Gewebezellen *in vitro*. *Acta Physiologica Scandinavica* **2**:145-188, 1941) in which the use of amino acids in animal tissue culture nutrients has been very carefully studied. While Fischer's results indicate that, in the absence of added vitamins and on a dialyzed plasma substratum, the amino acid formula of Bergmann and Niemann (On blood fibrin, a contribution to the problem of protein structure. *Jour. Biol. Chem.* **115**:77-85, 1936) is superior to that of Madden and Whipple, they agree with the present work in showing the prime importance of the amino acids.

showed the optimal total concentration to be about 1000 mg. per liter, about 68 times as high as for plant tissues (White, 102). The essential nature of these substances appears very strikingly. Cultures in one series in a nutrient lacking amino acids showed heart beat for only three days while in the corresponding nutrient plus amino acid heart beat was still evident after 32 days, with two renewals of nutrient. Similar results were obtained in other series. The solution finally settled on had the following concentrations in mg. per liter of nutrient:

dl-lysine-HCl	156	dl-isoleucine	104
l-leucine	156	l-arginine-HCl	78
dl-methionine	130	dl-phenyl-alanine	50
dl-threonine	130	dl-tryptophane	40
dl-valine	130	l-histidine-HCl	26

Concentrations of individual amino acids have not been varied but are based directly on Madden and Whipple's formula (60, 61). They will obviously require more careful study later.

Organic Catalysts: All cells apparently require for normal functioning a considerable, even a large number of organic materials, the optimal concentrations of which are so low that they are generally looked upon as catalysts rather than tissue constituents. These are the vitamins and hormones and certain related substances. Some cells apparently manufacture a large part of these substances themselves from simpler inorganic and organic materials; others can manufacture only a few. Thus, the wild-type of *Neurospora sitophila* supplies all its "trace" requirements except for biotin (Beadle and Tatum, 15; Tatum and Beadle, 86), root tips of tomato require external supplies of only two such substances, thiamin and glycine (or thiamin, pyridoxine and niacin) (Bonner, 16-18; Robbins and Bartley, 73; Robbins and Schmidt, 75-78; White, 103, 106, 107, 113). The vitamin and hormone requirements of animal tissues have been touched on by a number of authors (Burt, 22-24; Hamilton and Plotz, 45) but it is obvious that no clear-cut results can be obtained when organic complexes such as embryo juice, themselves sources of unknown amounts of many such substances, make up a large part of the nutrient. Thus one of the more complicated "synthetic" media, that of Baker (7) recommended for fibroblasts and epithelial cells, contains "vitamin A, some vitamin D, ascorbic acid, insulin and thyroxine". It contains no thiamin, niacin, pyridoxine, riboflavin, or biotin, to mention only the better known trace substances. We must

either assume that fibroblasts can manufacture these substances, that they are supplied in adequate amount in the peptone and serum provided, or that fibroblasts do not need them. We have no data on which to make a decision among these three possibilities, as regards any one of these, or of a considerable number of other substances.

In the absence of any experimental data it has seemed best to begin by supplying as many hormones and vitamins as may be *suspected* of being required, at concentrations which it is hoped will not be sub-minimal, and later to sift out the essential from the unessential ones by a series of group approximations. After some experiment these were provided in two solutions, one containing the B complex vitamins, the other a variety of other substances including vitamin A. These gave the following constitution per liter of nutrient:

B vitamins		inositol	0.5 mg.
thiamin	0.1 mg.	choline	5.0 mg.
pyridoxine	0.5 mg.	biotin	0.1 mg.
niacin	0.5 mg.	folic acid	0.001 mg.
riboflavin	0.1 mg.	ca-pantothenate	0.1 mg.
A vitamins and vitamin C:		ascorbic acid (vitamin C)	0.5 mg.
carotene (α 90%, β 10%)	0.1 mg.	glutathione	1.0 mg.
vitamin A	0.1 mg.	cysteine-HCl	1.0 mg.

Glutathione and cysteine-HCl serve as buffers against too rapid breakdown of vitamin A. These constitutions and proportions have not been subjected to experimental scrutiny. They will almost certainly require revision at some later date.

The respiratory intermediates, citrates, pyruvates, etc., and glucocides in general have been omitted in this work since evidence of their essential nature is too fragmentary to permit their inclusion on any rational basis at the present time.

Certain other matters which are not nutritional in nature also require attention. These are:

pH Indicators: Animal tissue cultures will endure long continued contact with nutrients at pH's from 6.6 to about 7.8, the optimum being about 7.2. This is best indicated by phenol red. The concentration given by Parker (67), 50 mg. per liter (50 ppm., 50 γ per ml.) is higher than necessary. Ten mg. per liter is enough. It is best introduced in the stock amino acid solution (see later) where it serves also as an indicator for the alkalinity of this stock. There seems to be no evidence of its being toxic at the concentration used (1:100,000).

Volume of Nutrient: Gey and Lewis give nutrient formulae in "drops" of each ingredient per tube. Lewis also specifies in some papers 1 ml. of nutrient. In my experience this is not enough. Two ml. is better. While this undoubtedly creates a greater *difference* in environment between cultures near the tip of the tube and those nearer the mouth, it gives considerably greater stability and less danger from accumulation of metabolic products.

Total Osmotic Value: Gey and Gey's solution (43) has a total osmotic value of 8.43 atm.. (freezing point lowering, calculated = —0.625° C.. Parker (67) gives the ΔT_f of Tyrode solution as —0.62°). It has been my experience with plant nutrient solutions that a rather wide range of osmotic values is tolerated (0.5 atm. to about 10 atm.) depending on a variety of other conditions, and that the optimum value is not necessarily the isotonic value (114). The optimum value will also vary with the ratios of certain ingredients in the nutrient, especially the ratio of carbohydrate to other electrolytes. Exploratory experiments showed that in a Locke-Lewis solution chick embryo tissues remain in good condition at osmotic values from 3 atm. up to 11 atm. when the ratio of ingredients is unchanged. The value of 8.4 atm., lying about in the middle of this range, may be considered suitable though not necessarily optimal. This question will be examined more closely later.

Gas Exchange: The continuous flow of nutrient over the tissues, the latter being exposed in the gaseous phase for 80 to 95 per cent. of each rotational cycle, furnishes ideal conditions for free exchange of gases between the tissue and its surroundings. The use of $NaHCO_3$ permits a considerable degree of buffering against too great fluctuations in CO_2 content and the presence of phenol red indicator makes it possible for the observer to follow such changes as do occur and renew nutrient whenever necessary. Atmospheric air has been used in all these experiments. Controlled gas mixtures with increased CO_2 and/or O_2 concentrations were given preliminary tests but did not prove markedly beneficial. This question must, of course, be studied more carefully later.

Supporting Substratum: Harrison in 1907 (47) introduced the use of clotted lymph as a combined nutrient and supporting mesh for the cultivation of animal tissue cultures. Burrows substituted blood plasma three years later (20). This has been standard practice ever since. Attempts to substitute such things as spider's web (Harrison,

47), cotton threads (Fischer, 40), silk veiling (Carrel, 25), etc., have had some success but have never replaced the use of blood plasma. Because of its unknown nutritive properties this is, of course, incompatible with the objectives set for the present work. Fortunately, if the glass of the culture vessel is *clean* and the cells are given an opportunity to become firmly attached thereto before being subjected to extraneous mechanical stresses such as the ebb and flow of the nutrient, this alone will serve as a fairly satisfactory substratum. Advantage has been taken of this fact in all the present work. If cultures are allowed to remain undisturbed in the tubes either for about a half-hour *before* adding nutrient, when the tubes are still plugged with cotton and fairly rapid drying takes place, or for about two hours *after* charging with nutrient and sealing with rubber stoppers before being placed in the rotor, the tissues will generally be quite firmly fixed to the glass. This varies somewhat with different tissues—heart muscle sticks well, skeletal muscle and amniotic epithelium fairly well, brain tissue very poorly, possibly because of its high fat content. Chick embryo tissues will survive in an unwarmed (room temperature) and unrotated tube for surprisingly long times. One set thus exposed for 44 hours resumed normal heart beat and growth as soon as it was placed in the incubator. Such long periods, however, give results which lack the uniformity of more carefully treated cultures.

Attachment, and subsequent migration and growth, although commonly good even on bare glass, is not invariably so. More *reliable* results may be obtained with a plasma clot, although neither survival nor growth is superior thereon to the best results obtained with uncoated glass. After considerable experiment, the following procedure was found satisfactory and was employed in many experiments as a basis of comparison.

Blood was drawn from the wing vein of a chicken in 10 ml. lots, using a chilled syringe and number 18 needle. Five ml. were introduced into each of two tubes containing 30 ml. of nutrient to which heparin (Connaught Laboratories) had been added to give a concentration of 1:80,000. These were centrifuged and the supernatant decanted into fresh tubes. This gives about a 1:10 dilution of plasma and about 1:100,000 heparin. This preparation will keep for many weeks in the refrigerator. Culture tubes were streaked with tissue as usual and laid with the tissue up. Two-tenths of a ml. of diluted

plasma were then added to each tube, being careful that the plasma should not touch the tissue. When a sufficient series of tubes were charged they were taken up one by one, the plasma carefully washed over the interior of the tube until it had all been wetted except the tissue streak. The plasma was finally allowed to come in contact with the tissue and was then flowed over the entire wet surface by a quick rotation (two to three complete turns) of the tube. Excess plasma was then drawn off with a pipette and the tube was laid on a flat rack, tissue again up. Clotting should be complete in 10 to 20 minutes and any excessively *thick clot* is thus away from the tissue, not covering it. The tubes were finally charged with 2 ml. of the desired supernatant nutrient, stoppered and placed in the incubator.

This procedure gives a final plasma-to-nutrient ratio of 1:100 yet furnishes a firm clot on which migration is regularly excellent. It is not liquified by 8-day chick embryo tissue.

Such a substratum has one pronounced advantage over bare glass. Re-attachment of small masses of tissue or single cells, which float free in the supernatant when cultures are first made up, is rare with glass but occurs frequently on plasma. The *coverage* is thus much more complete, fewer cells are lost on changing nutrient and the *amount* of tissue retained over long periods may be considerably greater than when plasma is omitted. Such cultures do not, however, survive for any longer periods than do those lacking plasma. Plasma does not appear to provide any nutrient materials which are not supplied in adequate amount in the synthetic amino acids and vitamins supplied in stock nutrients.

Discussion

These experiments have demonstrated that a very considerable degree of growth of a number of different embryonic tissues can be obtained in a synthetic nutrient. The results are, indeed, sufficiently good to justify the expectation that, with further refinement and extension, the method may eventually permit unlimited growth in fully known and precisely duplicable nutrients, as is already possible with plant tissue cultures. This is obviously necessary before a true science of animal tissue nutrition can be developed.

Unfortunately (or fortunately from another point of view) there is very little in the literature with which to compare these results. Levi's huge compendium (52) on morphology of cells in tissue culture

is illustrated almost exclusively with pictures of killed, fixed materials. None of his illustrations, with the exception of those of skeletal connective tissue and possibly pericardium, resembles at all closely the cultures illustrated in this paper. If we consider the types of tissue grown, it is possible to compare these cultures of heart muscle with those of Hogue (50) and the earlier ones of Lewis (55), but except for the confirmation of prolonged survival there is little resemblance either in procedure or in the results which these authors have considered of sufficient importance to enumerate. The skeletal fibroblast cultures differ so markedly from the classic Carrel picture (Ebeling, 36; Parker, 66; and others) as to evade comparison. The skeletal muscle cultures showed the semi-rhythmic contraction noted briefly by Pogogeff and Murray (69) and formed "muscle buds" similar to those illustrated by Parker (67, fig. 56 B). The flat sheets of amniotic epithelium may be compared with the germinal epithelium studied in Lewis' laboratory by Long (58) but I have found nothing in the literature comparable to the vesicular elements formed in these cultures, with the possible exception of Fischer's intestinal "organules" (39) and no description anywhere of the cellular motions which form the precursors of peristalsis. Only the nerve cultures find their counterpart in the classic investigations of Harrison (47) and his successors. From the points of view of procedure and of morphology there is, therefore, little to discuss. Here the facts themselves must suffice for the present.

From the point of view of nutrition, however, there is much that is pertinent. These studies have shown that the classic Ringer or Tyrode inorganic diluent can be very greatly modified without deleterious effect. Two modifications seem to me of especial importance. The substitution of sulfate and nitrate for some of the chlorides of Tyrode solution may not have any real significance, yet sulfur and nitrogen are both so obviously important and the radicals containing them are, in the nutrition of excised plant tissues, so clearly utilized that their incorporation into a nutrient seems to me very much worth while. Secondly, the heavy metals, especially iron, have been notably neglected. When considered at all iron is usually introduced as hemin. Baker uses 0.036 mg. hemin per liter of nutrient (7). This is 3×10^{-9} g. Fe ion per g. of nutrient. When one considers that the optimum for plant tissues is about 7×10^{-7} g. Fe ion per g. nutrient (115), about 200 times that supplied by Baker, and that with plant

tissues a variation of ten times either above or below this level is completely inimical to growth (112, 115) and when one considers further the known importance of iron in the whole process of cellular respiration (94), failure to study this factor adequately in the past is certainly a serious lacuna. It may be argued that the introduction of ferric nitrate (as has been done here) instead of hemin is not a suitable answer to the problem, yet it is well known that ferrous sulfate (as "Feosol") is the most effective source of iron in treatment of simple anemia and we have again the example of plant tissues which assimilate iron from the inorganic sulfates, chlorides or nitrates at least as effectively as they do from organic citrates or tartrates (98). Failure in these experiments to find evidence of the significance of zinc, manganese and copper has been interpreted as due to the presence either as impurities in other nutrient ingredients or as residues in the explants themselves, of sufficient traces of these substances to supply the minimal short-term requirements of the tissues studied. Copper, for example, is used in the purification of certain amino acids. More refined techniques will be required to elucidate these matters.

Carbohydrate metabolism has already been discussed in some detail. As regards the amino acids, Burrows and Neymann made some attempts to study amino acid nutrition in tissue cultures 28 years ago (21) and decided that they were toxic. A decade later (1926) Baker and Carrel came to a similar decision (8). Their data, however, do not justify such a conclusion. No one seems to have examined the question seriously in the past two decades.* This general failure to find evidence that amino acids were assimilated as such by animal tissue cultures, was at first thought by the present author to have been due to the high concentrations used by these earlier investigators, since the maximum dose tolerated by plant tissues is about 15 mg. per liter, while Baker and Carrel used a minimum dose of 60 and a maximum of 4400 mg. per liter. The present work indicates that this cannot have been the only explanation, since toxic effects did not appear with chick embryo tissues under these conditions until a level of about 1500 mg. per liter was reached. It must be remembered, however, that Baker and Carrel were using unknown and possibly sub-minimal amounts of the vitamins which may be necessary for the assimilation of amino acids. Furthermore, Baker and

*See footnote, page 275.

Carrel state only that they used "a mixture of 16 amino acids" but do not list either the amino acids used or the concentrations of each (8). Their results thus rest on such an incomplete foundation as to be inapt for direct comparison with this work.

In the field of vitamin nutrition, likewise, the comparisons possible are at best remote. Baker, in 1936 (7), employed "Vitamins A, B_1, B_2, C and D". Only ascorbic acid (vitamin C) was supplied in amounts designated by weight (0.85 mg. per liter), the others being in "units" ranging from 1000 per liter for vitamin A to 0.001 unit for B_2 and unknown amounts of vitamin D. Pyridoxine, niacin, pantothenic acid, inositol, biotin, cholin and folic acid were not knowingly supplied. Baker and Ebeling expanded this work somewhat in 1939 (14) adding a number of hormones (adrenalin, eschatin, pitressin, antuitrin), none of which is known to have any direct effect on generalized tissue growth. The vitamin components themselves were not modified. Burt (22-24) and Hamilton and Plotz (45) have added thiamin or biotin to complex culture media, studying their effects on *in vitro* growth of nerve cells but these are hardly comparable to the present study.

While there may be significant studies of this sort which have not come to the writer's attention, it seems clear that there is very little comparable literature on the subject. The 58 days survival of skeletal fibroblasts and 44 days for heart muscle reported here are equal to the 43-56 days survival reported by Baker and Ebeling (14) in a medium containing plasma, peptone and blood digest. It is the writer's belief that the investigation here described lends clear support to the conviction that potentially unlimited growth of animal tissues, *in vitro,* CAN be obtained in fully synthetic nutrients. Once that is done we can begin to study the nutrition of tissues and cells and eventually of organisms in a really rational way, and can hope to resolve such questions as the true rôle of thiamin in nerve metabolism, of iodine in thyroid function, of zinc in the activity of the pancreas, of potassium in the behavior of heart muscle, and others of like importance.

Summary

A procedure, based upon the roller-tube method of Gey and Lewis, and a fully synthetic nutrient incorporating current knowledge of the functions of vitamins, hormones, amino acids, oligodynamic inorganic ions, etc., has been developed for the cultivation of animal tissues *in vitro*. With this method and nutrient, and without the intervention of any unknown such as plasma, serum, or tissue juice, skeletal fibroblasts of 8-day chick embryos have been maintained in good condition for 58 days and heart muscle has continued to beat for 44 days, through repeated renewals of nutrient. Epithelium and nerve have been grown for shorter periods. It is believed that these results are sufficiently encouraging to lend support to the conviction that potentially unlimited growth of animal tissues, *in vitro*, can be obtained in a fully synthetic nutrient and that a true science of tissue nutrition can be based thereon.

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ON TREPHOCYTES AND TREPHOCYTOSIS; A STUDY ON THE ROLE OF LEUCOCYTES IN NUTRITION AND GROWTH*

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INTRODUCTION

Comparative and general hematology, and particularly that of invertebrates, have made only little progress during the present century when measured against the great advances in vertebrates, especially in mammals and man. However, the preoccupation of human and mammalian hematology with problems of defense physiology, in addition to distracting attention from other aspects of blood research, frequently had the effect that phagocytic or defensive functions were attributed to elements which possess no such qualities. Some of these misconceptions were indiscriminately applied in the study of lower animals and this in numerous instances resulted in obscuring the issues rather than elucidating them.

The present study deals with a certain type of leucocytes which are very common and possibly universal in the animal kingdom. In the invertebrates they frequently exceed in number the other cellular components of the blood. Due to the trends mentioned before, they were often confused with phagocytes although they are not phagocytic when fully developed. Their outstanding characteristic is the presence in the cytoplasm of nutritive inclusions which they carry and liberate throughout the body. These elements thus act as nursing cells, hence the term trephocytes was applied to them.

Before proceeding further, it is necessary to define well the terms as applied in this study in order to avoid misunderstandings which all too often result from indiscriminate use of the rich hematological nomenclature.

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The term *blood* is usually applied in general and invertebrate hematology when referring to body fluids and will be used here accordingly. *Blood-cells* designates elements capable of active or passive migration throughout the body; synonyms: free-cells, wandering cells, hemocytes. *Leucocytes* is used synonymously with white blood cells for all elements except the erythrocytes. *Lymphoidocytes* applies to leucocytes similar to the lymphoid series of higher vertebrates. Synonyms: hyalin leucocytes, amebocytes, phagocytes; the last two terms, however, are not too specific and are also applied to elements other than lymphoids. The lymphoidocytes are usually divided into two groups: (a) young, small forms with a relatively large and more or less spherical nucleus and little cytoplasm; non- or poorly ameboid or phagocytic; (b) larger forms stemming from the former, nucleus indented, incised, or reniform; cytoplasm abundant; highly ameboid and phagocytic. The first will be referred to as *lymphocytes* (synonym: hyalin leucocytes, stage I), the second as *macrophages* (synonym: hyalin leucocytes, stage II). The macrophages of invertebrates are often referred to as monocytes; as some investigators, however, claim a special position and character for the monocyte, the latter term should be reserved for vertebrates.

Trephocytes are leucocytes, generally larger than the lymphoidocytes in the same species. Their nucleus is relatively small, spherical; in the grown-up cell often fragmented into separate round units, sometimes pycnotic or missing. The protoplasm contains nutritive inclusions, often colored, and ranging in size from granules to single big globules filling the major part of the cell. They are known by many names, but most frequently as spherular leucocytes, eleocytes, or round cells. The function of these elements, which essentially consists in supplying nutrient and growth substances to various organs, tissues and cells, will be referred to as trephocytosis. The first term was suggested by Carrel (1924) for the vertebrate lymphoidocytes. It is seldom if ever applied to them, and consequently was adopted by the present writer to designate the elements dealt with here and for which it seems to be far more appropriate.

The terms *neutrophils, eosinophils* and *mastcells* (*basophils*) will be applied to elements closely resembling those of vertebrates, in appearance, specific granulations and function; but as invertebrates possess blood cells with specific inclusions which otherwise have little in common with the elements enumerated above, they will be referred to by circumscription, e.g. eosinophilic blood cells.

The conditions mentioned above indicate that specific granulations have very little value as criteria in a study ranging over as great a variety of groups as the present one. Though admittedly of importance in classifying the leucocytes of a given species, they sometimes are of doubtful use even within these limits (e.g. the early stages of eosinophils are claimed to contain basophilic granules) and definitely fail when we cross the species' boundaries. Thus, in the rabbit the granules of the neutrophils are eosinophilic, in the guinea-pig some eosinophilic, others basophilic; in Amphibia the neutrophils often exhibit no granulations. The problem with which we are concerned is primarily that of *form and function* of the white blood cells. Consequently only the totality of both morphological and physiological aspects can serve as guide in determining the nature of a given element.

The extent of the present study requires limiting ourselves to representatives of the following groups: coelenterates, echinoderms, annelides, crustaceans, insects, molluscs and tunicates. Other groups from which trephocytes are reported will be dealt with at the end together with a brief reference to vertebrates.

Coelenterates

Though lacking a circulatory system and a mesoderm proper, coelenterates are known to possess blood-cells, usually referred to as wandering cells. Allmann (1872, quoted from Billard) was apparently the first to have seen amongst them granular cells in the extremities of *Obelia* stolons. A number of succeeding authors observed similar elements in other Hydrozoa, in Scyphozoa and Ctenophora; they noted ameboid movement in some of these cells and their glandular nature, as well as high refringency and occasional coloration of their granules.

Billard (1904) gives a detailed description of these cells from a series of hydroids. He found them mainly in the ectoderm and sometimes in the endoderm and describes them as relatively large, more or less ameboid elements with a small, usually eccentrically located nucleus, which is often hidden by the abundant granules. The latter are coarse, sometimes attaining the size of spherules up to 4.6 μ in diameter and evolve from finer granules found in the younger cells. They do not stain with eosin and appear to be neutrophilic or distinctly basophilic in nature. A chemical analysis in one species did

not give a clear indication of their character except for water solubility and iodophilia, which may suggest glycogen. He observed their great abundance in regions of rapid growth and in the reproductive zooids. As to their nature, he at first thought they contained reserve material, but ultimately decided on an excretory function.

Kollmann (1908) confirmed most of the findings of Billard but did not agree with him on the excretory character of the granular cells. He tended rather to consider the granules as being secretory products.

Loeb (1916) assumed that the colored endodermal cells of *Tubularia* accumulating at the end where a polyp is to regenerate give off substances which cause polyp formation. Runnström (1929) gives a further description of these elements in *Clava squamata*. They originate in the endoderm, whence they migrate into the ectoderm. Mucicarmin stains them red, which suggests the presence of a mucin; the granules stain partly blue and partly red with Mallory-Azan. Runnström calls these elements "reserve cells" (Reservezellen) as he considers them to be carriers of reserve substances. He observed their accumulation in the regeneration blastema and saw therein an expression of their trophic function.

My own observations on *Tubularia sp.* (to be published) confirm and further extend the observations of Runnström and some earlier investigators of these elements. They appear as large pear- or goblet-shaped glandular cells in the endoderm (fig. 1a) and contain rather coarse granules of a reddish color. Various staining reactions and their behavior towards fat solvents suggest that the granules contain protein and phospholipin; some of them blacken after osmic-acid treatment. In some individuals small amounts of glycogen were detectable in the cells, not associated, however, with the granules. With Wright's blood stains the latter exhibit a variable reaction ranging from slight eosinophilia to moderate basophilia.

Histological observations indicate that these cells frequently become rounded when still in the endoderm and are emitted into the enteric cavity. Here they may undergo a twofold course of development: (a) they assume the appearance of moruloid granular elements (fig. 1b, c) or (b) the granules fuse and the cell transforms into a more or less homogenous, largely basophilic sphere, a so-called "pseudo-cell" (fig. 1b′, c′). In both kinds the nucleus degenerates and becomes incorporated into the cytoplasm. This suggests a release of

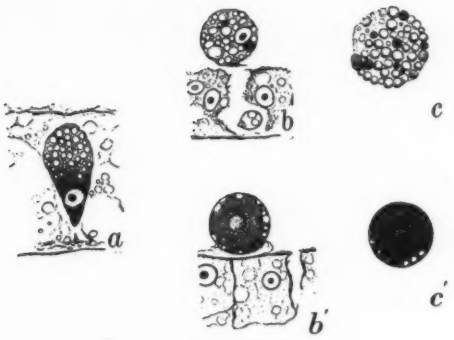

FIGURE 1
Trephocytes from a single piece of a regenerating stem of Tubularia sp.; ca. 500 x. (Original)

thymo- and ribonucleic acids from the nucleus, where they are normally present (Caspersson and Schultz, 1940) into the cell body. Similarly, basophilia of the cytoplasm or of the inclusions indicate the presence of ribonucleic acid (Caspersson and Schultz, 1939; Brachet, 1940 etc., and others). Assimilation of the nucleus by the cell body and basophilia of the cytoplasm and of the inclusions are also common amongst trephocytes of other groups suggesting that similar substances may be expected in these cells in general. Nucleic acids are essential in protein synthesis and their derivatives have a growth-promoting effect, which well agrees with the nature of the trephocytes.

In *Tubularia* these cells show signs of ameboid movement when still in the endoderm, but no indications of it are forthcoming after their release and particularly not from the pseudo-cells. The granular type shows a tendency to form aggregates and there also appears to occur a fusion of pseudo-cells.

As suggested by some earlier investigators, these elements are concerned with various growth phenomena. They are released in great numbers from the endoderm into the enteric cavity during regeneration and are observed to accumulate in the region of the prospective polyp formation where their substance is apparently utilized by the regenerate. It is evidently these cells which Loeb surmised as having a formative function.

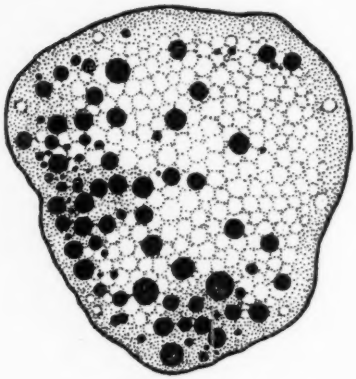

FIGURE 2
Egg of Tubularia sp. with numerous pseudo-cells; 200 x.
(Original)

The trephocytes accumulate in great numbers in the sexual individuals. Many "pseudo-cells" are found in the egg (fig. 2) and are gradually incorporated into its cytoplasm; some, however, persist into the larval (actinula) stage. The engulfment of these elements by the growing egg was already known to early investigators (Balfour, Metschnikoff and others). The views on their nature are much divided, some seeing in them abortive oogonia, others nursing-cells of the egg, their nuclei or those of the nursing cells.

In addition to the elements described, ameboid phagocytic lymphoidocytes are reported from coelenterates. According to Runnström, like the trephocytes they stem from the endoderm. Besides this common origin, however, there does not appear to exist any genetic relationship between these two groups of cells.

The outstanding characteristics of trephocytes, viz: large size, lack of ameboid and phagocytic capacities, presence of considerable amounts of nutritive material and disappearance of the nucleus, are already well expressed in the coelenterates. If one may judge from conditions in the mammalian erythrocyte, the loss of the nucleus suggests that the trephocytes are highly specialized cells even in this primitive group.

Echinoderms

To Geddes (1880) we owe the first detailed description of blood elements in echinoderms. He distinguished two groups in sea-urchins: (1) highly ameboid colorless cells, developing from smaller forms provided with a flagellum, and (2) less ameboid elements containing in the cytoplasm coarse granules of a mahogany-brown or sometimes a green or red color. He considered the differently colored granulocytes as variants of the same type and observed color changes in alcoholic extracts of their pigment, when exposed to the air or upon addition of acids. He surmized that the pigment has some relation to respiration. His classification of the blood elements, which holds good for all echinoderms, remains fundamentally unchanged up till today. Geddes' two groups correspond to our classification into lymphoidocytes and trephocytes and will be referred to accordingly. Cuénot (1891a, 1891b) assumed from studies on representatives of the main groups of echinoderms that the lymphoidocytes take up nutrient substances originating from the intestine, transform them into granules and thus become trephocytes (his "mahogany-brown" and "mulberry-like corpuscles"). He maintains the granules to be albuminoid or fatty in nature. They are carried into all the organs by the trephocytes and released and utilized by the tissues. In some species the trephocytes are assumed to change again into lymphoidocytes at the expense of some of their granules.

C. Saint-Hilaire (1897) draws a sharp line between the lymphoidocytes which, according to his observations, are phagocytic, and the trephocytes, lacking these qualities. He submitted the granules of the latter to a thorough investigation and found the colorless ones to be composed of lecitin and protein, and the red ones (Geddes' and Cuénot's "mahogany-brown") to contain pigment, lecitin, protein and an osmiophilic substance. The trephocytes were found to be most numerous in and around the intestinal wall but not exceptionally as abundant in the genital organs as expected. He also observed that in the body cavity the lymphoidocytes ingest trephocytes. As to the function of the latter, he was at a loss.

Kollmann (1908) stresses the large size of the trephocytes of various echinoderms when compared with the lymphoidocytes (fig. 3). The granules were found to be basophilic, the nucleus "invisible" and is not usually shown in illustrations. He did not observe transitional

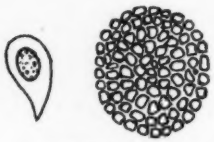

FIGURE 3
A lymphoidocyte and a trephocyte of Paracentrotus lividus; 1300 x. (After Kollmann)

stages between the trephocytes and lymphoidocytes as Cuénot did. Kindred (1924) found numerous trephocytes ("amoebocytes with spherules") in the regenerating test and assumed that they "probably carry nutrition to the cells . . . where repair is going on rapidly".

The pigment of the trephocytes attracted early attention. MacMunn (1885), who named it echinochrome, concluded that it is an oxygen carrier. More recently Cannan (1927) and others suggest that echinochrome is an "activator" rather than a "carrier" of oxygen, a view which is widely though not universally accepted.

Annelides

Amongst early investigations on the blood of oligochaete annelides those of Cuénot (1891a) are the most comprehensive. He maintained that their leucocytes are liberated chloragogue cells (chloragocytes). These are pear- or club-shaped elements covering the digesting portion of the alimentary canal and the adjacent blood vessels. They contain greenish granules which Cuénot considered albuminoid in nature and as evolved from the nutrients of the alimentary tract and the blood, a view expressed earlier by Claparede (1869). After becoming liberated, the chloragocytes were maintained to give off their granules into the coelomic fluid, diminish in size, become ameboid and thus transform into lymphoidocytes. Later (1898) Cuénot radically changed his opinion and gave up his views on the trophic nature of the chloragocytes in favor of an excretory conception.

Rosa (1896) proposed the term eleocytes (or oleocytes) for the granular, non-ameboid elements, on account of the oily nature of their inclusions. He denied, however, that these are liberated chloragocytes, as claimed by Cuénot and others. He was first to describe another type of large, non-ameboid cells which he called mucocytes, owing to their content of mucin.

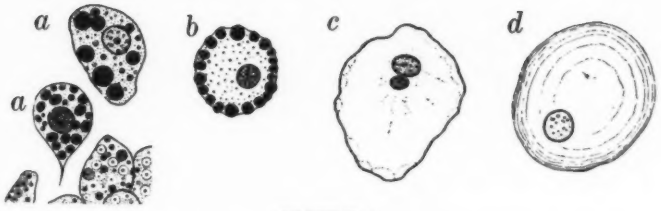

FIGURE 4
Trephocytes of Eisenia foetida; a,a, young forms; b, mature stage (eleocyte); c, old stage (mucocyte); d, "enigmatic cell"; 800 x. (Original)

Kollmann's (1908) conceptions do not differ widely from the later views of Cuénot, viz: that the granular leucocytes (including the eleocytes) evolve from lymphoidocytes. He confused the issue, however, by identifying both groups and by claiming phagocytic properties for what clearly appear to be eleocytes. This confusion is even more evident among later investigators.

The writer (1927, 1931) revived some of the early views of Cuénot on the trophic nature of the chloragocytes. Their granules were first believed to be taken up and carried by the lymphoidocytes into all the regions of the body, but in the later study it was established that the chloragocytes themselves carry the nutrients. Of great support to the conception of the trophic nature of these elements was the observation that they supply nutrients to the sex products, and migrate in great numbers into the growing ovarial eggs, whose deutoplasm is derived from the chloragocytes. These observations were later confirmed by Bell (1943).

Further problems were elucidated by studies in vivo, in vitro and by differential staining (Liebmann 1942b). The granulated leucocytes of various authors, the eleocytes and the mucocytes of Rosa were found to be successive developmental stages of liberated chloragocytes (fig. 4). Based on studies on oligochaetes and other groups, the term trephocytes was proposed for all these elements, which in oligochaetes were found to contain fat and protein, lecitin, glycogen, mucin and guanine, the latter a growth substance according to Hammett (1938). To the group of trephocytes also belong the so-called "enigmatic cells" or "linocytes" which represent mucocytes with a wrinkled cell wall, a fact easily confirmed in vitro (unpublished observation). Investigations by the latter method further revealed that the lymphoidocytes ingest portions of the trephocytes or the

granules released by them and assimilate them as other cells do. This observation elucidates the origin of granules in the lymphoidocytes of *Eisenia* and also helps to explain the repeated confounding of the latter with the trephocytes, when granulations are taken as sole classification criteria. In most oligochaetes studied, the trephocytes were found to be numerically superior to the other main group: the lymphoidocytes. Their granules were invariably basophilic, which suggests the presence of ribonucleic acid. In the adult cell the nucleus breaks up into several units or, as in *Tubularia,* it often disappears in the cytoplasm and the cell assumes the aspect of a moruloid or more homogenous sphere.

The trephocytes were observed to carry their substances into wounds and regenerating parts (Liebmann 1942a). While this takes place, and when the animal is sexually mature, production of ova is suspended as the trephocytes, which normally supply nutrients to the ova, now supply these substances to the wound and the regenerate. Conversely, during the period of oviposition, regeneration is inhibited owing to the fact that the trephocytes are taken up by the developing eggs (Liebmann 1942c). Thus the correlation between sexual reproduction and regeneration was explained and a similar mechanism suggested for the correlation between sexual and asexual reproduction. By reducing the diet and thus keeping the production of trephocytes on a low level, it is possible to suppress growth and sexual reproduction in *Eisenia foetida* for a long period (unpublished observations). In short, the correlation of various forms of growth was found to be controlled by the trephocytes. Considering that similar correlations exist in widely separated groups of animals, their elucidation by studying the physiology of the trephocytes seems to be promising.

In *Eisenia* the trephocytes form aggregates resembling some lymphoid organs or rather a fat body of insects, but actually serving regeneration and thus justly considered regeneration organs (Liebmann 1943). There are two such organs: a "head" organ and a "tail" organ, composed of two kinds of modified trephocytes: "head trephocytes" and "tail trephocytes", a phenomenon reflecting regional specificity. The extent and gradient of each of these aggregates correspond to, and apparently determine the limits and gradients of head or tail regeneration. The trephocytic aggregates are functionally polarized and act in one direction only, which coincides with, and

seems to determine, the direction of regeneration. Partial removal of the head organ inhibits regeneration and occasionally causes formation of monstrosities (Liebmann 1945).

Conditions in polychaetes appear to be very similar to those in oligochaete annelides. Williams (1852) gives the first accurate description of trephocytes in polychaetes (and in the animal kingdom in general) stressing their very large size, tendency to aggregate, frequent lack of a nucleus and presence of "oil vacuoles and granules". Succeeding investigators found similar elements in other polychaetes and Claparede (1868, 1873, quoted from Romieu) observed them forming a fat body before maturity, which he believed gives rise to the sexual elements. Similarly, Cuénot (1891a); Caullery and Mesnil (1898) noticed an abundance of trephocytes prior to sexual maturity and the disappearance of their granules during this process, and assumed thereby their vitellogenous function.

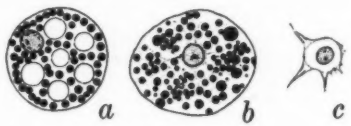

FIGURE 5
Leucocytes of polychaetes: a, trephocyte of Sabella pavonina, 650 x (from Kollmann); b, c, a trephocyte and a lymphoidocyte of Amphitrite ornata, 500 x. (Original)

Kollmann (1908) describes leucocytes, often poorly ameboid and with a fragmented or pycnotic nucleus, containing fine granules usually eosinophilic, in some species neutro or basophilic; in the latter case they resemble mast-cells. He believed that these cells derive from lymphoidocytes. In addition, he found in many species large non-ameboid and non-phagocytic elements with albuminous and fatty inclusions (fig. 5a). He could not link these cells with lymphoidocytes and called them "adipo-spherular cells". In some species they were very numerous, the lymphoidocytes comprising only a small fraction or apparently lacking; he also saw the so-called "enigmatic cells".

Romieu (1923) confirmed in the main Kollmann's findings but disagreed with his designation adipo-spherular cells, offering instead the established term of eleocytes to stress the similarity of these elements in both oligochaetes and polychaetes. Contrary to Kollmann,

he considered the trephocytes as derived from the leucocytes with finer and usually more basophilic granules. Like earlier investigators (including Kollmann) he observed diminution of the trephocytes and disappearance of their granules during sexual maturity.

My own investigations (unpublished) on *Amphitrite ornata* reveal conditions similar to those in oligochaetes. There are two main groups of leucocytes in this species: trephocytes and lymphoidocytes (fig. 5b, c) and there are apparently no transitional forms between the two. The trephocytes sometimes make up 50% of the total and exhibit all the characteristics peculiar to these cells. I found them in the connective tissue and muscle, where they appear to give off to the surrounding cells their granules, so far identified as of a lipid and protein nature. With the lymphoidocytes, they accumulate in the region of an inflicted wound and liberate their cell contents into the cicatrix. Vivo observations indicate that their granules are also ingested by the lymphoidocytes. Prior to the release of the granules the nucleus appears pycnotic and the cells often disintegrate during this process.

The trephocytes represent liberated chloragogue cells in species possessing this kind of tissue. In forms devoid of it, the cardiac body, an organ found in the dorsal blood vessel and regarded as an inverted chloragogue tissue, is widely considered to be the hemocytopoietic center for the trephocytes. Thus, in the annelides, the lymphoidocytes and trephocytes represent two groups of cells already distinct at the moment they are liberated into circulation.

Crustacea

Early students of crustacean blood observed only one type of leucocytes in it. Thus Haeckel (1857) described ameboid cells, with numerous coarse granules, which he believed to represent fat. Later investigators began to distinguish two types of leucocytes: hyaline (or finely granular) and granulated. Löwit (1891) thoroughly examined the leucocytes of the Crayfish and found that their inclusions vary in size from fine granules to coarse droplets and are neutro- to eosinophilic. They represent, in his opinion, a globulin or a closely related substance. Cuénot (1891a, 1895) based on studies of numerous species, distinguished in the hyaline forms two stages corresponding to our lymphocytes and macrophages and maintained that the granulocytes develop from the hyaline cells by elaboration of gran-

ules, a view sustained up to the present. The granulocytes, sometimes quite large, were found to be non-phagocytic and when fully developed often non-ameboid. He observed that their nucleus breaks up into several fragments, the granules fuse and escape into the body fluids where they are often ingested by the lymphoidocytes, observations which were amply confirmed by succeeding investigators. The granules or spherules, usually yellowish or greenish in color, were assumed to represent a ferment, a view which was retracted in the second study.

Hardy (1892) apparently lumped the hyaline and the granular leucocytes of the Crayfish together into one group: his "eosinophile corpuscles", but in addition described two more types from this species: "explosive corpuscles" and "basophile cells". He found the explosive corpuscles to outnumber the eosinophilic elements by 3:1. They explode or disintegrate within seconds after withdrawal from the body, a phenomenon characteristic of trephocytes in general, especially of the older forms. Because of their fragility, he could not study them in vitro and his deductions from smears are misleading. In vivo he observed them to be of a flattened ellipsoid shape and densely filled with granules. The third type of elements, his "basophile cells", were only numerous in animals "poisoned with certain substances". They originate from similar cells covering the arteries and already described by Haeckel. Later investigators (Cuénot, Kollmann) agree that the basophile cells are not normally found in the blood, or at least are very scarce.

Kollmann (1908) points out that the inclusions of the so-called eosinophilic elements are usually only slightly acidophilic or neutrophilic, sometimes basophilic or metachromatic. They represent, in his view, an albuminoid and play a role in the nutrition of the organism, as was demonstrated by his numerous experiments and observations on *Carcinus maenas*. During fasting the number of cells and their granular content decreased markedly and rose again after abundant feeding. Several days before and after moulting, there was a considerable diminution in their numbers allegedly due to the fact that the animals do not feed during this period. Parasitised individuals and those developing sexual products similarly showed a fall in the number of granulocytes. Contrary to general opinion, Kollmann maintains to have observed these cells with phagocytosed india ink.

Betances (1922) took up the problem of the "explosive corpuscles"

and maintains that these elements (since Hardy described under various names such as: thigmocytes, rhagioplasts, etc.) together with the granulocytes represent one group of cells, the differences observed being an expression of various phases of a secretory function. His views do not seem to have been contradicted since then. He maintains that the cells have a trophic and restorative function and that the granules contain an albuminoid and sometimes mucin. Brettschneider (1938) found the blood cells to form a syncytium beneath an inflicted wound, and to give off their inclusions in a manner much resembling conditions described by the author in annelides. Several other writers also mention accumulation of granulocytes near wounds.

FIGURE 6
Blood cells of the trephocyte type from Artemia salina, 1500 x.
(After Lochhead and Lochhead)

Recently Lochhead and Lochhead (1941) have submitted the blood of *Artemia* to a thorough investigation. The blood elements were found to be predominantly granular or spherular, varying in size and shape (fig. 6) but probably originating from one type. The inclusions were observed to be amphophilic. Their chemical nature could not be ascertained except for a possible presence of fat and protein. The cells disintegrate in a varying degree when exposed to air and within the body give off their inclusions in circumstances resembling those observed in oligochaetes. In female individuals in particular, the granulocytes were found with discharged inclusions, a phenomenon which the authors connected with the growth of the eggs. In and beneath inflicted wounds they form compact syncytial aggregates and release their granules in a manner resembling conditions near the ovaries.

The great similarity of conditions, both morphological and physiological, in Crustacea with those in other groups described earlier is evident and justifies classifying their granular and spherular cells as

trephocytes. In the species investigated they appear to make up the majority of the leucocytes. They are often termed eosinophils on account of the frequent tinctorial reaction of their inclusions and erroneously considered by some as analogs of the vertebrate eosinophil. However, they have very little in common with the latter either in appearance or in function.

Insects

The tendency of trephocytes to form aggregates appears to be very common. Sometimes these are relatively large but transitory as in the cases reported in connection with wound healing, regeneration and growth of the eggs. In *Eisenia,* however, there is a permanent aggregate in the head whose elements are replenished from the circulating trephocytes. Such aggregates or tissues seem to be much more common than hitherto assumed. They have sometimes been compared with the fat body of insects. The term "fat body", as Wigglesworth (1939) rightly remarks, is a misnomer for, like the trephocytes, it contains also protein, glycogen and other substances. The fat body of Crustacea is apparently also derived from blood cells according to Jäger (1935) and others. In insects the close connection between the circulating elements and those forming the fat tissues is generally conceded; it will therefore be necessary to deal with both in the present chapter.

Graber (1871), first to study thoroughly the blood of insects, found its elements to be composed of (a) ameboid and relatively rare forms; (b) round or elliptic granular cells. The inclusions of the latter are frequently colored and of a fatty nature. Cuénot (1891a, 1896) claiming for the insect leucocytes a development similar to that of Crustacea, found the small, ameboid cells (Stage I) in frequent division; the inclusions of the granulocytes albuminoid and eosinophilic, their nucleus often fragmented or pycnotic. Kollmann (1908) again points out that the reaction of the inclusions varies according to species, being usually neutrophilic.

Hollande (1909, 1911), based on extensive studies, distinguishes five types of leucocytes: (a) proleucocytes (maintained to be the stem cells of the others); (b) phagocytes; (c) granular leucocytes; (d) leucocytes with spherules; (e) oenocytoids. The first two correspond to our designation of lymphocytes and macrophages and together with the granular leucocytes are claimed by Hollande to be

practically universal in insects. The granular leucocytes (fig. 7a, b, c) are described by him as oval or spherical, relatively large, with granules varying in size, and of an acido- to basophilic reaction. In Lepidoptera the granules fuse into a single drop filling the whole cell and giving it the appearance of what is usually termed a signet-ring cell (like fig. 7e). He believes the leucocytes with spherules

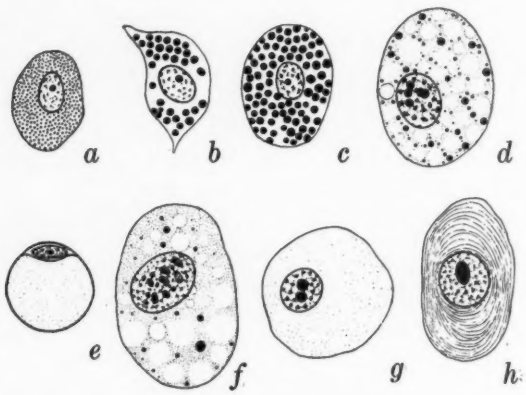

FIGURE 7
Various blood elements of insects: a-c, granular leucocytes; d, spherular leucocyte; e, signet-ring cell; f, adipoleucocyte; g, oenocytoid; h, "enigmatic cell"; varying magnification (from Hollande)

(fig. 7d) to be identical with those described by Kollmann in Crustacea and molluscs. The spherules were observed to develop from eosinophilic granules by formation around them of a basophilic substance or fat, with scattered eosinophilic granules persisting through the mature cell. The numerous spherules often give the cell a moruloid appearance; less frequently there are only a few inclusions and then they are usually large. In Coccides, finally, the cell is occupied by a single sphere giving it a signet-ring appearance (fig. 7e). The leucocytes with spherules were found to be large, spherical to ovoid and non-phagocytic in the mature stage; their nucleus small, sometimes double. Their inclusions are given off into the blood like those of the granulocytes. When exposed to the air they often explode or disintegrate. In *Pyrrochorus apterus* he observed elements which he

calls "adipoleucocytes" and considers as free floating fat cells (fig. 7f). They are, however, hardly different from the spherular cells (compare fig. d with f). The oenocytoids, as described, are very large, non-phagocytic cells with a relatively small nucleus (fig. 7g). Their cytoplasm is clear or very finely granulated and acidophilic. With these elements he also groups what are often called "enigmatic cells" which he observed in a single species (fig. 7h).

There are several inconsistencies in Hollande's endeavor to separate the granular from the spherular elements. From his own description it appears that we have here morphologically and functionally very similar elements appearing, according to species, in one or the other form and, where they occur, the spherular cells developing from granular elements. From my own observations on *Tubularia,* annelides and particularly on tunicates, I believe myself justified in considering the granular, spherular and signet-ring cells as trephocytes. As to Hollande's oenocytoids, they may represent trephocytes which have discharged their inclusions and are similar to the mucocytes of the oligochaetes. In one case at least he found several (remaining?) spherules in the oenocytoids.

Ensuing investigators further subdivided the insect leucocytes. Poisson (1924), for example, distinguishes amongst the phagocytes young and old forms and with essentially the same modification the subject is presented by Wigglesworth (1939). On the other hand, there is growing realization amongst recent investigators that there are essentially two main groups of blood elements originating from a common stem cell: (a) phagocytes and (b) non-phagocytic elements, carrying granular or spherular inclusions (Lazarenko, 1925; Blaustein, 1935; Murray and Tiegs, 1935, and others). There is practical unanimity amongst investigators that the elements of the second group are numerically prevailing except perhaps during metamorphosis in those species where phagocytes are known to play an important role in disposing of the degradation products of cytolysis.

It is generally agreed that both the blood cells and the elements of the fat body are derivatives of the mesoderm and in very young larvae are indistinguishable from each other. Graber (1871) maintained that the fat body is formed from free blood elements. To stress this close relation, Wielowiejski (1886) introduced the term "blood tissue" to include the free blood-cells, the fat body and their derivatives; Graber (1891) uses "hemosteatic tissue" in the same sense.

Schäfer (1889) considered the fat body to represent sessile blood-cells and Berlese (1900, 1901) holds a similar view. Krüger (1898, quoted from Kremer, 1917) was first actually to observe transitions from white blood cells to those of the fat-body in *Tenebrio* and Chrysomelids. The imaginal fat body of Muscids arises from typical leucocytes according to Janet (1907). Poisson found in aquatic Hemiptera and Lazarenko (1925) in *Oryctes* larvae, that the fat body grows through life by inclusion of leucocytes containing fat and there are several indirect indications that the process may be much more common than assumed. In the wings of Coleoptera free leucocytes become aggregated to form a fat body, (Kremer 1917, 1925) and the same was observed in the moth *Ephestia* by Zeller (1938). In *Pyrrhochorus,* according to Hollande (1911) and in Aleurodids according to Weber (1935) the fat cells are free. Altogether the organization of the fat-body may range—depending on the species—from loose aggregations to compact masses of cells covered with a delicate membrane (Snodgrass, 1935). In the latter case the same range of organization is observed when proceeding from the larval to the imaginal stage (Berlese). All this and the fact that during metamorphosis the elements of the fat body again become free (at least in the Holometabola) only stress the impossibility of drawing a dividing line between the free trephocytes and those of the fat body.

The main function of the insect trephocytes is the elaboration of nutritive substances and their liberation into the blood and tissues of the body (Cuénot, Hollande, Kremer, Wigglesworth and others). Lipids and protein appear to be practically universal both in the free and the integrated elements; glycogen is commonly found in the fat-body. Pigments and uric acid are also quite frequent; the latter is considered to be of endogenous origin and to represent a degradation product of protein (Wigglesworth, 1939, 1942, and others).

Lazarenko (1925) describes accumulations of large leucocytes, of the type which form the fat-tissue, around implanted foreign bodies after the phagocytes have cleared the débris and formed a capsule around them. These "round cells" give off portions of their cytoplasm, which is believed to indicate some chemical activity. A similar phenomenon was observed by the same author (1928) in wounds caused by cauterisation. Ries (1932) believes that leucocytes (type not specified) accumulating around wounds aid the healing process;

and he observed but little or no phagocytosis. There are apparently no studies on the possible role of leucocytes in regeneration.

In contrast with the paucity of investigations dealing with the function of free trephocytes, there is a considerable number of studies on the fat-body.

Starvation of the larva causes depletion of the reserves from the fat cells in *Tenebrio* (Kollmann 1909), *Dytiscus* (Kreuscher, 1922) and in mosquitoes (Wigglesworth, 1942). These are restored when feeding is again resumed. The reserves are partly used up during moulting (Poyarkoff, 1910). But it is during metamorphosis that the action of the fat cells is most evident. The accumulated substances become released into the blood and are apparently utilized in the extensive building-up processes taking place at this time. The cells may largely survive this process (Berlese, 1900, 1901) or, as in the Hymenoptera and higher Diptera, dissolve completely (Perez, 1902, 1910, 1911, and others).

In the adult, the fat body is often considerably larger in the female than in the male, e.g. in mosquitoes (Liebman, unpublished observations). There is also a close correlation in these species between the egg production and size of the fat-body: with the advance of cold weather in autumn, oviposition ceases abruptly and at the same time the fat body increases considerably in size. The latter is used up during hibernation when the insects as a rule do not feed (Liebman, unpublished studies).

There are some isolated claims for Lepidoptera and Coleoptera that the fat cells phagocytose débris of histolysed tissue (Poyarkoff, 1910; Hufnagel, 1918; Murray and Tiegs, 1935). Such a statement, when based exclusively on fixed material, should be viewed with caution. The prevailing opinion is that the inclusions of the fat cells are of endogenous origin.

Amongst the various elements grouped together with the blood-cells, the oenocytoids and oenocytes show a close functional resemblance to the trephocytes. The oenocytoids become very numerous during moulting and metamorphosis (Poyarkoff; Wigglesworth, 1933), which supports the writer's contention that they may represent trephocytes which have discharged their inclusions. Some authors (Hollande, 1914; Kremer, 1917, 1925) believe that the oenocytes also are transformed fat-cells and their close relation to the fat body is usually conceded. There is general agreement that they

discharge some substances into the blood and that their function is closely related to growth. Glycogen, protein and lipids have been demonstrated in some species (Poisson; Hollande, 1914) but several other substances, amongst them hormones and enzymes, are suspected (see Wigglesworth, 1933, 1939). Their function—probably variegated in such a large and heterogenous group as the insects—as well as their relation to the similar oenocytoids remain to be worked out in detail.

The trephocytes of the insects and their function are not different from those of the lower forms hitherto described. The trephocytic aggregates of the latter, best known from the annelides, are homologous to the fat body and similarly represent accumulations of cells and substances to be utilized during periods of intensified growth, like reproduction and regeneration. The regular development of these formations into fat bodies in insects and Crustacea is doubtless linked with the presence in these groups of cycles of explosive growth known as metamorphosis and moulting.

Molluscs

Leydig (1850), studying *Paludina vivipara,* mentions two kinds of leucocytes from its blood: (a) round elements, and (b) cells with processes (pseudopodia). A similar classification, usually into granular and agranular leucocytes, is followed by investigators closely succeeding him and practically up to the present. Leydig also describes large, clear cells, later named after him and having a small parietal nucleus and inclusions believed to be of a calcareous nature. These elements were found in locations where connective tissue cells are present in higher animals. Following investigations revealed the presence of genuine connective tissue cells and J. Kollmann (1877) found distributed amongst them an additional type of cells, containing granules, frequently colored, which he called "round cells" (Rundzellen). There is much confusion about all these elements, partly because there appear to exist transitional forms between them.

Cuénot (1891a) is not very explicit as to the blood elements of molluscs. He maintains that there exists only one kind of them: "amebocytes". They often contain fine granules which he believes represent ferments. In gasteropods and lamellibranchs he found elements frequently large, with coarse, usually colored inclusions of protein and fat; in *Doris tuberculata* these cells contain one or a few

droplets of the same composition (signet-ring or droplet cells). All the elements with inclusions are poorly ameboid. Studying the connective tissue (1892), he describes in addition to the Leydig cells coarsely granular elements (J. Kollmann's round cells), stresses their lack of movement and similarity with the mast-cells of vertebrates in tinctorial reaction. His later contention (1900) that they represent excretory cells is based on their reaction to injected stains and does not withstand criticism. De Bruyne (1896), investigating phagocytosis, enumerates seven kinds of leucocytes, amongst them those of the spherular type (réticulés or vacuolisés). He did not study the latter and his classification is confusing, considering that he represents functional variants as distinct types.

List (1902), in a very careful study, carries further the investigation of the round cells. He describes them as large, spherical or oval, non-ameboid elements with a small nucleus. They contain, often in the same cell, granules, spherules and vacuoles. Some of these inclusions are osmiophilic and are generally considered as nutritive in nature. The cells have a tendency to aggregate. They often greatly resemble the granular leucocytes on one hand, and there are also transitional forms between them and the Langer's vesicles (= Leydig-cells; Fleming's mucus-cells) on the other hand. The latter, as described, are very similar in some species to the mucocytes of earthworms but in others they contain granular inclusions indistinguishable from the round-cells. In some cases no round cells were detectable in the connective tissue and List believes that the Langer vesicles assume their function. There are indications that there exists a genetic relationship between the granular cells of the blood and those of the connective tissue (the round-cells) and the Langer vesicles. List claims this only for the last two, maintaining that the round-cells develop from the Langer vesicles. Concerning their function, he holds the view that both carry nutrient substances; during sexual reproduction he observed profound changes in them.

M. Kollmann (1908) examined representatives of the main mollusc groups. He describes two stages of hyaline, ameboid elements (lymphoidocytes) encountered in the blood of all groups but the cephalopodes. The smaller divide actively and give rise to larger, highly phagocytic cells on one hand and to granular elements on the other, a contention made earlier by Cuénot. The granulocytes are present in the blood of most species and are ameboid. In lamellibranchs,

where he studied them more closely, their nucleus is often constricted or fragmented and the granules acido- or amphophilic. He considers these cells as having a nutritive function, as he observed a sharp decline in their numbers in poorly fed animals and after hibernation. In the blood of *Mytilus edulis* he also found non-ameboid, spherical elements containing granular, eosinophilic reserve substances. He applies to them the term "Rundzellen". These are also common in the connective tissue where they attain a much larger size. He considers the Rundzellen as the ultimate developmental stage of the granular, ameboid leucocytes (fig. 8a, b, c). In the connective tissue of other lamellibranchs, and in molluscs in general, he observed elements very similar to the Rundzellen and calls them "spherular cells" (cellules sphéruleuses). They are large, non-ameboid and similar to the fat-cells of insects. Their inclusions are coarsely granular to spherular, sometimes neutro- but mostly basophilic (fig. 8 d, e). The inclusions in his opinion represent reserve substances and the cells were observed to become rare in hibernating animals. Kollmann also briefly describes the Leydig cells in Amphineura as containing acidophilic, albuminoid granules representing a reserve substance.

In contrast to List, there is much confusion in Kollmann's work owing to his lack of discrimination in terminology and the apparent attempt to classify the elements according to the size of their inclusions. His distinction between Rundzellen and spherular cells is unjustified and other authors use these terms (coarsely granular or spherular "Körnerzellen" and "Rundzellen") synonymously. What seems to be clear is that we have here a developmental continuum. According to Kollmann's own description, the Rundzellen are mature

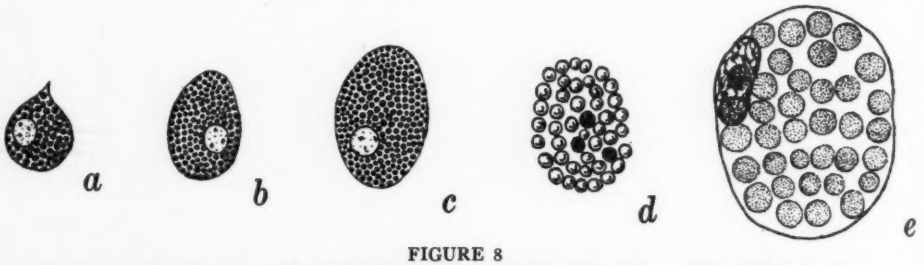

FIGURE 8
Leucocytes of molluscs: a-c from Mytilus edulis; a, granular leucocyte; c, "Rundzelle"; b, form transitional between a and c; d, spherular cell of Chiton marginatus; e, similar cell from Helix pomatia; all 1300 x. (After Kollmann)

granular leucocytes and this probably is the case with his spherular cells. The fact that both the granular leucocytes and the spherular cells diminish in number during starvation lends some support to the latter view and suggests that there may exist a similarity in their function also.

Succeeding investigations were almost exclusively concerned with the phagocytic-eliminatory or phagocytic-digestive function of the leucocytes. In the latter category the studies of Yonge (1926, 1928) are of considerable interest. He observed "phagocytic cells" take up formed and dissolved nutrients from the surface and lumen of the alimentary canal and carry them into the connective tissue where they are digested. Part of the digestion products are given up to the epithelial cells of the gut and the remainder transported to the Langer-vesicles (Leydig-cells) and there stored. The type of leucocytes performing this function was not determined nor the way they store the substances in the Leydig cells. Takatsuki (1934) is somewhat more explicit; examining the blood from the heart of the oyster he found two main types of leucocytes: hyaline and granular, both kinds ameboid and phagocytic. The granules were found to be neutrophilic and to contain fat. The relation of the blood granulocytes to those of the connective tissue and the latter themselves were not studied.

Despite a considerable amount of study on the molluscan blood, our knowledge of their trephocytes is very fragmentary. From the account of Cuénot (1891a) it appears that coarsely granular, spherular and signet-ring types are present in the blood of molluscs. M. Kollmann found spherular non-ameboid cells in the blood of some species only. However, according to all accounts, their presence in the connective tissue of molluscs appears to be universal. Here they are predominantly basophilic and sometimes similar to vertebrate mastcells. According to M. Kollmann, the practically identical Rundzellen represent ameboid granulocytes of the blood which migrated into the connective tissue and there reach maturity and full size. A similar phenomenon was observed by the present writer in a tunicate and will be dealt with in the next section.

Morphologically, the granular, spherular and signet-ring leucocytes of the molluscs correspond to the trephocytes of other groups. They are claimed to develop from lymphoidocytes, the transitional stages being ameboid and (according to Takatsuki) phagocytic. The mature forms, however, are to all accounts apparently non-ameboid and

non-phagocytic. Their condition during sexual reproduction, starvation and hibernation suggests that their function is probably trophic.

Tunicates

Early observations on the blood elements of tunicates were superseded by the thorough investigations of Cuénot (1891a). He distinguished among them: (a) "typical amebocytes" from which all the others are claimed to develop; (b) "amebocytes with fat", usually very numerous; (c) "amebocytes with a reserve vacuole" containing a single large drop of an albuminoid or oily nature and, in contrast to the previous, non-ameboid; (d) various colored amebocytes. He maintains that the elements with reserve substances carry and liberate them into various tissues and organs. This passing of nutritive material from cell to cell explains the low content of protein in the blood of Tunicates and other invertebrates, an opinion voiced earlier by Krukenberg (1882). Kollmann (1908) observed that the inclusions of the leucocytes vary in tinctorial reaction from acidophily to basophily or metachromasy. He ascribes excretory or phagocytic properties to elements hardly capable of such functions and considered trophic by Cuénot and others. A summary of earlier studies on tunicate blood is given by Seeliger (1893-1911).

Spek (1927) studied the blood-elements of *Clavellina lepadiformis* with the aid of vital stains, primarily neutral-red and Nile-blue sulphate. He found the most common forms to be ameboid ones, usually containing a single colorless droplet (signet-ring or droplet cells) which stains electively with those dyes. The droplets were found to be composed of protein with a probable admixture of lipids. During adverse conditions, usually in the autumn, great numbers of the droplet-cells migrate into the stolon, form aggregates and together with other ameboid elements make up the interior of the winter buds. When a new organism develops from the buds, all its interior organs are asserted to be formed from the cell-aggregates. In regenerative processes the droplet cells are claimed to play a similar role. They also surround the eggs and give up their inclusions to them. Summarizing, Spek maintains that these elements have a double function: (a) as carriers of nutrients and (b) as "totipotent" cells capable of differentiating into various organs. The latter conclusion, however, is in sharp contrast with what is known of the specialized nature of these elements in tunicates, as well as in other groups, and seems un-

warranted by the limited character of the technic applied by him. The general opinion is that the various organs are re-formed from mesenchymal elements similar to, or identical with, the non-specialized lymphocytes.

George (1939), summarizing his studies on numerous tunicates, distinguishes, in addition to lymphocytes and macrophages: (a) vacuolated cells, some with nutritive inclusions; (b) nephrocytes, elements with vacuoles containing purine bases; (c) cells with colored granules, in some cases identified as carotenoids. Like earlier investigators, he observed that a large proportion of the blood-cells serve as nursing cells for the tissues.

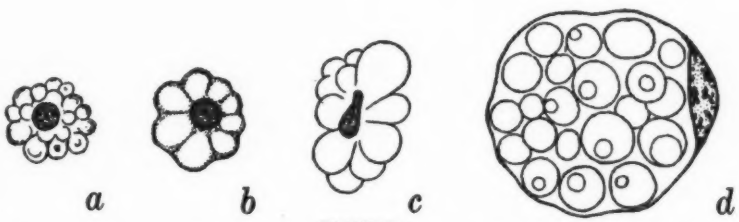

FIGURE 9
Trephocytes of tunicates: a-c of Perophora viridis; d, from the connective tissue of Styela partita; 1500 x. (Original)

My own investigations, now in progress, suggest that the granular, spherular and signet-ring cells are trephocytes which appear in one or the other form, depending on the species examined or the age of the cell, and also change accordingly the chemical aspect of their inclusions. They exhibit the general characteristics common to all trephocytes: considerable size, relatively small nucleus which is often fragmented or pycnotic, and poor or lack of ameboid movement in the developed stage.

The following species were studied: *Perophora viridis, Styela* (*Cynthia*) *partita,* and *Molgula manhattensis.*

In *Perophora* three kinds of trephocytes can be distinguished, their size, cellular and nuclear characteristics and the numerous transitions amongst them suggesting that we actually deal here with different age classes of the same elements. Consequently we will designate them as young, mature and old forms (fig. 9a, b, c).

The young forms are about the size of the largest lymphoidocytes

(6-8 μ). They contain coarse, resistant, chromophobe granules of a yellow to orange color. As a rule they do not blacken with osmic acid though they sometimes exhibit a brown or blackish tint leading over to the next type. These are apparently the orange cells of George (1926).

The mature forms reach a somewhat larger size than the young ones (6-10 μ). Their nucleus is sometimes elongated or fragmented. The inclusions as a rule are larger than in the previous stage, spherular or drop-shaped and, in life, of a yellowish- to dark-green color. They blacken easily with osmic acid; Best's carmine and the sputum test reveal the presence of heavy masses of glycogen in the cytoplasm but not in the droplets; in some cases mucicarmin indicates the presence of mucin. These are the green cells of George and other authors.

The third type is frequently not much larger than the previous though some may reach almost double the size. In all cases, however, the inclusions are large. In life they are colorless and do not usually blacken with osmic acid. They are strongly eosinophilic and with Mallory stain golden orange. Glycogen is sometimes detectable in small amounts. The nucleus is often elongated, fragmented or compact and pycnotic. The latter is the case in the largest forms, which

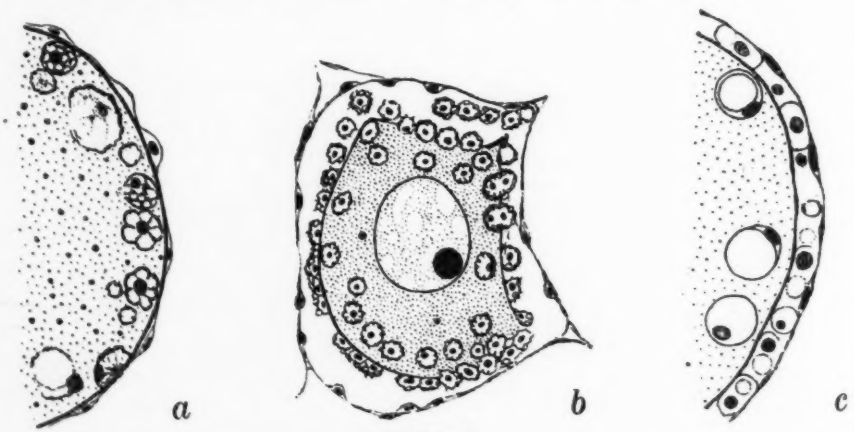

FIGURE 10

Trephocytes in tunicate eggs: a, of Perophora viridis, 350 x; b, young egg of Styela partita with some trephocytes in the process of immigration; 350 x; c, trephocytes in a mature egg of Molgula manhattensis, 1000 x. (Original)

show signs of dissolution with accompanying disappearance of staining capacity and cyto-chemical characteristics.

All these elements are found throughout the body. Within the tissues and organs the young and mature forms appear to move through ameboid action; the old forms do not show signs of ameboidism. The young and the mature forms give off their inclusions into the surroundings, the cells when they survive assuming the appearance of mucocytes or oenocytoids.

Ovarial eggs examined early in July contain great numbers of mature trephocytes (fig. 10a) usually near the periphery but often as far as the nucleus. The appearance of fixed material indicates that the majority give off their inclusions and disintegrate, the substance of the whole cell being assimilated by the ovum. The nuclei appear to resist longest and are observed scattered all over the egg. The presence and fate of the trephocytes in the growing egg is a further and very reliable indicator of their function.

The presence of cells in the tunicate egg was already known to early investigators and gave rise to various speculations. More recent studies (summarized by Seeliger) consider them to be follicle- or testa-cells, the latter term indicating their similarity with elements found in the testa (tunic). However, their function and hematic origin remain unknown. Even Harvey (1927), who rightly concluded that they contribute a fraction of the egg yolk, is unaware of their nature and calls them testa-cells.

In contrast to the spherular trephocytes in *Perophora,* those of *Styela* are of a coarsely-granular type. There are several kinds of these; their exact genetic relationship remains to be determined. In the nutrition of the egg a graded variety is involved, all showing similar cyto-chemical traits: their granules are basophilic to metachromatic, do not blacken with osmic acid, are positive to Best's stain and mucicarmine.

Early in its development young ameboid elements of this type invade the ovary. Part of them insinuate themselves between the individual eggs and their follicles, forming a kind of secondary follicle around them (fig. 10b). They penetrate in great numbers into the ova, the process continuing until the yolk platelets become quite distinct. Those within the egg (and to a limited extent those surrounding it) seem to grow along with the egg with the result that up to the stage of yolk formation there appears a regular size-propor-

tion between the egg and its trephocytes. It is, however, of greater significance that the trephocytes within the egg develop cyto-chemical qualities not observed in those outside it. This suggests a certain functional nexus between the ovum and the ingested trephocyte. The greater part of the ingested trephocytes disintegrate and are assimilated by the ovum, as is also the case in the third species to be described.

Elements similar to those in large eggs are very common in the connective tissue (fig. 9d), where they appear to attain a further increase in size and look similar to mast cells of vertebrates. It is worth while mentioning here that the latter also reach extreme size in the tissues.

Conditions as described for *Styela* are found to a varying degree in the ovaries of the two other species. In *Perophora* there is only an accumulation of trephocytes around the ova and follicle formation appears to be exceptional. In *Molgula,* on the other hand, a regular follicle is formed by the trephocytes. Here they are of the signet-ring type (fig. 10d), the most common form encountered in this species. They develop in a way similar to that described by Hollande for occides. Both in *Molgula* and in *Perophora* a limited size relation also exists between the ovum and the ingested trephocytes and the latter develop cyto-chemically in a different way than those outside the egg.

Owing to considerable seasonal variations in the composition of the blood, the exact proportion of the trephocytes cannot be ascertained without an elaborate study. At the beginning of July they made up about 50-60% of all leucocytes in *Perophora* and *Styela.* In *Molgula* their proportion was even higher.

The so-called nephrocytes are in all respects identical with the trephocytes, except that instead of the nutritive inclusions they contain purine bases (for references, see George 1936, 1939). It is assumed that they collect these substances from the blood and tissues. My own though limited observations suggest that these substances are of endogenous origin representing products of the cells' own metabolism. This is also well established for insects where elements of the fat-body or hemocytes transform into urate cells as a result of accumulation of their own degradation products.

Some of the early investigators of tunicates (Krukenberg and others) believed that the pigment of their blood-elements has a respir-

atory function. Henze (1911, 1912, 1913) discovered a vanadium chromogen and free sulphuric acid in the moruloid (spherular) green cells of several ascidians. Though the notion on the respiratory function of the chromogen was apparently later disproved by several investigators, no definite function has been found for it so far (see Webb 1939).

The trephocytes of the tunicates investigated by the present writer apparently represent the three morphological types encountered in the animal kingdom: granular, spherular and signet-ring cells. The chemical nature of their inclusions and the circumstances in which they are released leave little doubt as to the function of these cells. Their transformation within the egg suggests that they not merely supply substances into regions of growth, but also enter a close physiochemical interaction with the object they nurture. This recalls the condition in the earthworm *Eisenia* where "head" and "tail" trephocytes develop apparently due to some regional factor of the body. In addition to their nursing function, the trephocytes of tunicates may have some relation to respiration and, as in other groups, to pigmentation. The latter two aspects, however, do not fall within the realm of trephocytosis.

Conditions in Other Invertebrates and Vertebrates

Trephocytes are reported from other animal groups also. They were observed by several early investigators in the mesenchyme of various sponges. Cotte (1904, quoted from M. Kollmann) found them to be ameboid and sometimes phagocytic to carbon. Their granules are considered albuminoid in nature and, according to Cotte, are basophilic in the mature stage. He also seems to have observed these elements giving off portions of their cytoplasm into the surroundings and to degenerate under adverse nutritional conditions, or when the organism was injured. Kollmann (1908) describes large, apparently non-ameboid cells with a small, often pycnotic, nucleus; they contain droplets of an ampho- to basophilic substance. He compares these elements with the spherular leucocytes of other species but is undecided as to their function.

Galtsoff (1925), studying restitution in *Microciona prolifera,* found the mesenchyme composed of what he terms "archeocytes", i.e. hyaline and granular leucocytes. The granular elements, comprising the majority, have inclusions ranging from 1-6 μ in diameter and corre-

spond to the granular, spherular and signet-ring cells of other species. They usually contain a red pigment, the inclusions being greenish-yellow. The archeocytes are the elements most active in the process of restitution of the organism and their various forms are claimed to represent metabolic phases. At the other extreme, Wilson and Penny (1930), studying the same species, distinguish a number of types, amongst them granular "gray cells" and "globoferous cells," with a single large inclusion. According to George (1941), the granules of the former represent a reserve substance which is used up in regeneration.

In the small but hematologically very interesting group of sipunculids, Cuénot (1891a) describes amebocytes with fine albuminoid granules; some of these elements contain in addition colored, spherular inclusions of a fatty nature. The latter are often very numerous so that they arrest the ameboid movement of the cell, which usually assumes relatively large size. Kollmann (1908) finds that the elements with spherular inclusions are neither ameboid nor phagocytic, and that their inclusions are neutro- to basophilic. The same author describes hyaline and granular elements from the blood of myriapods, the granular hemocytes being considerably larger in size and in number than the first group. The granules are more or less acidophilic. He found transitional forms between the two types in diplopods; they are apparently lacking in chilopods.

The blood of scorpions and spiders has been studied only little. Kollmann (1908), in agreement with earlier investigators, distinguishes two kinds of elements with inclusions in scorpions: granulated and spherular. The first are oval with a small nucleus and neutrophilic granulations. The second are somewhat larger, their inclusions basophilic, the nucleus small or missing. Their number varies greatly within the same species and is particularly high in animals whose blood was in the course of renewal. He did not find transitions between these elements and the other groups and believes that they originate from the connective tissue.

In spiders, Cuénot (1891a) distinguishes: (a) finely to coarsely granular amebocytes and (b) large forms containing, in addition to granules, also prisms of a proteid reserve substance. The latter are said to evolve from the first group and to move slowly without emission of pseudopods. Kollmann (1908) adds hyaline leucocytes to this classification but his account of the other elements and their role

during moulting hardly elucidates the confusion existing at this point from earlier investigations. Millot (1926) observed a great increase of "vacuolated" leucocytes in the week preceding and that following the moult, with a corresponding decrease of the hyaline and granular leucocytes. Similar conditions are reported by Deevey (1941). She found a great increase of "leberidocytes" during moulting. These elements are very similar to the mucocytes or oenocytoids of other invertebrates. They contain glycogen and are held responsible for the increase of this substance in the blood during moult.

Amongst the vertebrate blood elements, the mast-cells (basophils) bear the closest resemblance to the trephocytes of invertebrates. They are poorly ameboid and non-phagocytic. The tissue mast-cells, whose genetic relationship to those of the blood is conceded, at least for the lower vertebrates, attain extreme size as do the invertebrate trephocytes in general and those of the connective tissue in particular; in this situation their nucleus is small, sometimes fragmented or pycnotic. It was recognized early by Ehrlich that the mast-cells are concerned with the nutritional condition, a view which became widely accepted. They have a tendency to form aggregates and are known to give off portions of their plasma with granules, a process best known as clasmatosis; in some situations the cells disintegrate completely. The granules were repeatedly claimed to contain fat, protein and mucin, substances also common in the trephocytes of invertebrates; their basophilia suggests the presence of ribonucleic acid.

A comparison with the invertebrates suggests, however, that the basophils represent a numerically and functionally vestigial cell-system. The process of evanescence of the blood mast-cells is conspicuous even within the vertebrates, where they are claimed to decrease from a relatively high percentage in the Amphibia to an insignificant fraction in the mammals.

Some functions characteristic of the trephocytes and mast-cells are to a certain degree exhibited by the vertebrate elements of the lymphoid series, notably by the lymphocytes. Some investigators see in the latter specialized cells, whilst others consider them as both morphologically and physiologically pluripotent elements. Considering that the lymphocytes are apparently also capable of phagocytosis, the writer tends to view their trophic function (such as clasmatosis or the fact that they are ingested by the egg) as only one of the several activities of an unspecialized cell. This opinion is further supported by the observation that other primitive elements exhibit a similar multiplicity

of functions. For example, the follicle cells of Amphibia actively phagocytose degenerating ova whilst in the case of the growing egg they give off portions of their plasma into it or are phagocytosed by it, thus serving to a limited extent as trephocytes (Liebman, 1945).

Notwithstanding a great amount of investigation, the problem of the trephocytes and trephocytosis in the vertebrates is not clearly understood and still highly controversial. Even a cursory presentation of it would greatly exceed the limits of the present paper and must be reserved for a future study.

Discussion

A review of literature and the writer's own studies on *Tubularia*, annelides, insects and tunicates reveal that, in practically all invertebrates, two main groups of leucocytes are found: lymphoidocytes and trephocytes.

There are admittedly other types of leucocytes amongst the invertebrates but they appear sporadically in a few groups only. Ciliated urns are found in sipunculid worms. In the latter, as well as in the polychaetes, elements which are intermediate between erythrocytes and trephocytes are common. I studied them in *Cirratulus grandis* (unpublished observations); they show a fixed, elliptical size, protoplasmic structure and hemoglobin content, like the erythrocytes; on the other hand, the small or fragmented nucleus, the numerous lipid inclusions and pigment granules resemble those of trephocytes. A few genuine eosinophils are sometimes encountered in earthworms near inflicted wounds (Liebmann, 1942b). They are reported in similar circumstances by Lazarenko (1925) from an insect where they make up 1 per cent of all leucocytes. I have never found them under normal conditions in earthworms. Whether they occur more widely in insects is problematic.

In numerous instances the trephocytes appear to evolve from young lymphoidocytes which in this process develop nutritive inclusions, lose their ameboid and phagocytic faculties and grow considerably in size. The fully differentiated forms are apparently neither ameboid nor phagocytic.

The contention going back to Cuénot, that the trephocytes always develop from lymphoidocytes appears, however, to be erroneous. I have found no evidence for it in *Tubularia* nor in numerous oligochaetes studied. In both cases the trephocytes, when liberated from the hemopoietic tissue (i.e. at the moment when they actually be-

come blood-cells), are neither ameboid nor phagocytic. In the oligochaetes the trephocytes are formed along a particular section of the mesoderm, which only serves to stress the special character of these elements. Similarly, in polychaetes, they are claimed to be liberated by a special organ, located in the main blood-vessel, the so-called cardiac-body. Crustacea and insects too possess hematic tissues along the main blood vessels which possibly serve as formative loci for trephocytes. Kollmann (1908) similarly points out that he could find no transitional forms between the trephocytes and the other blood elements in echinoderms, sipunculids or scorpions.

Nor is the difference between the lymphoidocytes and the trephocytes a matter of origin only. The two types differ widely both in morphology and function. In contrast to the lymphoidocytes, the trephocytes are large, usually surpassing by a wide margin all other blood-cells in size; their nucleus is small, often fragmented, pycnotic or missing. The function of the lymphoidocytes consists in the main in phagocytosis and elimination of noxious or foreign matter; the trephocytes produce, distribute and release nutritive and growth substances into the blood, tissues, or cells. They often penetrate into the latter, where they become assimilated. Their function, however, is not merely nutritive in the usual sense but they sometimes appear to enter some close physio-chemical relation with the recipient and become modified in a way which reflects regional or organ specificity.

In animals whose blood plasma is poor in dissolved nutrients, which probably comprises most invertebrate groups, the trephocytes are the main or sole carriers of these substances. This does not lessen their importance in those groups, like the insects, which usually contain a high percentage of dissolved nutriment in their blood. In all cases, the activity of the trephocytes is most pronounced in various phenomena of growth such as: wound healing and regeneration, moulting and metamorphosis, and the growth of ovarial eggs.

Taking all the previous points into consideration, the conclusion is reached that, for a relatively complete and correct understanding of the function of white blood cells, it is necessary to supplement the phagocytic theory with a theory of trephocytosis. In the main the former would apply to lymphoidocytes, the latter to the trephocytes. Considering that the trephocytes are numerically superior in many annelides, in crustaceans, insects, tunicates and some smaller groups of invertebrates, it is permissible to assume that in them trephocyto-

sis is antecedent to phagocytosis. This apparently holds for all groups as far as various growth phenomena are concerned.

In conclusion, it is of interest to note that, owing apparently to their large size, the trephocytes were discovered earlier than the lymphoidocytes (by Jones, 1846). Williams (1852) gives a very apt description of them in a series of invertebrates, and his illustrations are particularly revealing. It appears that he also saw ameboid lymphoidocytes but considered them to be distorted elements, as their nature was then unknown. In conformity with the general conception on the nutritive character of the blood, and due to the fact that fat or albuminoid inclusions were found in the trephocytes, several succeeding investigators anticipated their character and Cuénot (1891a) gave in many cases a correct interpretation of their function. Under the influence of the theory of phagocytosis he later reversed his earlier views and went to the extreme of seeing in practically every kind of leucocyte a phagocyte or an element serving excretion. This tendency prevailed too often for almost three decades. Kollmann (1908) admittedly did not hold such extreme views as Cuénot but, by relying too exclusively on criteria derived from specific hematological stainings of vertebrates, he reached some erroneous general conclusions regarding the nature of the invertebrate leucocytes. It is only towards the end of the twenties that a gradual turn towards a more balanced conception on the leucocytes began to make its way.

Summary and Conclusions

This study reveals the presence in invertebrates of two main groups of leucocytes: lymphoidocytes and trephocytes.

Both types differ widely from each other in morphology and function. In contrast to the lymphoidocytes, the trephocytes are large, usually surpassing in size all the other blood-elements; their nucleus is small, often fragmented, pycnotic or missing. Whilst the function of the lymphoidocytes consists mainly in phagocytosing and eliminating noxious matter of endo- or exogenous origin, the trephocytes produce, transport and release nutritive and growth substances into the blood, tissues and cells. They are frequently observed to move into the latter and the whole trephocyte, including its nucleus, becomes assimilated by the recipient element. In animals with a low content of dissolved nutrients in the blood they appear to be the main or sole carriers of trophic matter. Their function is most con-

spicuous in various manifestations of growth, such as: wound healing and regeneration, moulting and metamorphosis, and in the growth of ovarial eggs. In contrast to the mainly catabolic function of the lymphoidocytes, that of the trephocytes is primarily anabolic.

It is frequently claimed that the trephocytes evolve from unspecialized lymphoidocytes which, during this process, develop inclusions, losing pari passu their ameboid and phagocytic properties. However, in oligochaetes and in polychaetes they are produced in particular sectors of the mesoderm or within special organs, which only serves to stress their distinct character. When released from these centers, the trephocytes already show their specific characteristics such as large size, cellular inclusions and lack of ameboid and phagocytic properties. In coelenterates the trephocytes also appear to develop independently of the lymphoidocytes and there are indications that this is the case in some other animal groups.

For a comprehensive, correct understanding of the function of the leucocytes in general the occurrence of trephocytes must be taken into consideration, particularly since they appear to comprise the majority of the white blood-cells in numerous annelides, in Crustacea, insects, tunicates and in some smaller groups of invertebrates. The theory of phagocytosis should therefore be supplemented by a theory of trephocytosis, which is the aim of the present study. Considering the numerical prevalence of trephocytes in the aforementioned groups, one may assume that in them trephocytosis exceeds phagocytosis. This probably holds good for many other invertebrates whilst they are undergoing various forms of growth.

The trephocytes frequently contain colored matter which is assumed to have some relation to respiration. So far this seems to have been established only for echinochrome. The pigment of trephocytes is often directly or indirectly responsible for the animals' coloration. Trephocytes or their derivatives are claimed to produce in some cases substances not strictly nutritive, e.g. wax and hormones in insects.

The mast-cells (basophils) of vertebrates resemble trephocytes most closely both in morphology and function. They seem, however, to represent a rather vestigial cell-system.

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THE INFLUENCE OF DIET ON MITOTIC DIVISION IN THE INTESTINAL EPITHELIUM OF THE MOUSE

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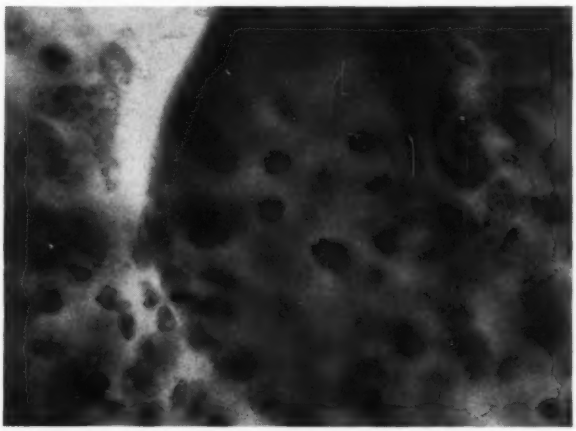
During the course of an investigation of the effect of a bacterial (S. marcescens) polysaccharide on rapidly dividing sarcomas (1) it was found that all dividing cells, whether normal or neoplasmic, are damaged by this agent. In the normal young adult mouse, the intestinal epithelium is one of the most actively dividing tissues of the body, and five to seven or even more, mitotic figures may be observed in the core of each villus at all times (text fig. 1). When S. marcescens polysaccharide was injected into tumor-bearing animals, intestinal epithelium was also sometimes severely damaged, as shown in fig. 8 of the previous paper (1). An attempt was made, therefore,



TEXT FIGURE 1
Acetic orcein smear preparation of intestinal epithelium of young albino mouse on normal diet. Note dividing cells. (x 900)

to suppress mitotic activity in the intestinal epithelium of the mouse, with the hope of overcoming adverse gastric symptoms during polysaccharide therapy.

Theoretically, at least, a part of the intense mitotic activity in the epithelium should be due to repair processes following erosion by rough food wastes. It was our hope that administration of very bland or fluid diets, such as milk, would avoid wearing down and consequent repair of the intestinal epithelium, thus reducing the number of dividing cells present.

In the first experiment, young (57 and 63 days of age) healthy albino mice, both males and females, were employed. These were matched litter mates reared in our laboratory from Carworth Farms stock. The animals were divided into three groups. One group received a rough diet consisting of oats, raw peanuts and compressed food in pellet form (Purina dog chow, Gaines' dog pellets, and Rockland mouse food); the second group of mice received nothing but milk; and the third was given water, but no food. When the first of the starved animals succumbed, all the rest of the animals were sacrificed, and slides were made from the intestinal epithelium of each animal by means of the acetic-orcein method, which preserves each villus intact.

Counts of dividing cells were then made from five fields of ten villi each from each of the experimental animals. The results are shown in Table I.

The average count, ranging between 53.6 to 66.4 mitoses per field in the animals maintained on rough diet fell to as low as 6.2 per field in the starved animals. The highest mitotic average in the groups of animals maintained for four days on water only was 15.8. But the number of mitoses per field in animals fed solely on milk remained nearly as high as in the group of animals fed on regular diet, and was somewhat higher in males than in females. These figures suggest that mitosis depends not on erosion and repair of the tissues, but on the nutritional state of the animals.

To test this hypothesis further another experiment was conducted in the same fashion, but the weights of all animals were carefully noted at the beginning and the end of the experiment. The animals were sacrificed at the end of four days when all those maintained on water were near the point of death, but none had succumbed. The results are shown in Table II and are almost identical with those

TABLE I

INFLUENCE OF DIET ON MITOTIC DIVISION IN THE INTESTINAL EPITHELIUM OF THE MOUSE

Animal no.	Age in days	Sex	Duration of exp.	Diet	Number of Mitoses					Average
					Field 1	Field 2	Field 3	Field 4	Field 5	
335.	63	Male	4 days	Control	43	62	55	65	53	55.6
340.	57	Female	4 days	"	64	59	77	62	70	66.4
341.	57	Female	4 days	"	57	57	52	48	54	53.6
342.	63	Male	4 days	"	52	48	59	62	58	55.8
336.	57	Male	4 days	Milk	59	55	67	55	66	60.4
337.	57	Female	4 days	"	54	51	48	40	46	47.8
338.	57	Male	4 days	"	57	57	52	48	54	53.6
339.	57	Female	4 days	"	34	48	40	51	37	42.0
331.	63	Female	Died on 4th day	Water only	6	7	8	0	10	6.2
332.	63	Male	Died on 4th day	"	13	14	14	9	11	12.2
333.	62	Female	4 days	"	20	9	11	4	8	10.4
334.	62	Male	4 days	"	22	15	11	17	14	15.8

TABLE II

INFLUENCE OF DIET ON MITOTIC DIVISION IN THE INTESTINAL EPITHELIUM OF THE MOUSE

Animal no.	Age in days	Sex	Duration of expt.	Weight gain or loss (grams)	Diet	Number of Mitoses					Average
						Field 1	Field 2	Field 3	Field 4	Field 5	
463.	54	Male	4 days	—0.75	Control	56	70	51	55	49	56.2
464.	54	Male	4 days	+2.30	"	41	59	53	70	64	58.4
465.	54	Female	4 days	—	"	51	57	47	53	44	50.4
466.	54	Female	4 days	+1.50	"	63	57	58	53	49	56.0
469.	54	Female	4 days	—2.80	Milk	49	54	47	51	56	51.4
470.	54	Female	4 days	—3.00	"	46	47	42	55	50	48.0
471.	54	Male	4 days	—2.50	"	50	65	60	52	57	56.8
472.	54	Male	4 days	—3.50	"	70	54	66	52	52 (5 deg.)*	58.8
467.	54	Female	4 days	—5.30	Water only	27 (7 deg.)*	37 (6 deg.)	21 (9 deg.)	32 (14 deg.)	17 (20 deg.)	26.8
468.	54	Male	4 days	—8.20	"	2 (9 deg.)	2 (5 deg.)	5 (3 deg.)	3	5 (2 deg.)	3.4
521.	50	Male	4 days	—6.0	Amigen	65	67	79	67	57	67.0
522.	50	Male	4 days	—6.1	"	41	51	45	49	48	46.8
523.	50	Female	4 days	—4.2	"	59	100	99	74	91	84.6
524.	50	Female	4 days	—4.1	"	67	62	59	77	67	66.4

*Degenerating mitoses.

revealed by Table I. The animals which fed solely on milk lost a little weight, and there was a slight drop also in the number of mitoses, as compared with the controls.

The evidence was not conclusive, however, that mitosis in intestinal epithelium is not a repair function, since milk forms a curd which might conceivably exert some mechanical damage to the epithelial lining during passage. To obviate this uncertainty, still a third type of experiment was carried out, in which the animals were given no solid food or milk, but a synthetic mixture of amino acids, glucose and salts, in the form of a protein hydrolyzate known as Amigen.* The hydrolyzate was administered in amounts proportionate to the dose suggested by its manufacturers (Meade, Johnson and Co.) for humans, which resulted in a daily mouse ration of 0.5 cc. of 5% solution. It was dissolved in distilled water and proferred to our experimental animals in their drinking bottles, but the surface tension of the water was so greatly lowered that the fluid immediately leaked from the tubes. However, when the solution was made up in tap water it was easily retained in the bottles, but the mice refused to take any appreciable amounts of the solution, probably because of its sour taste and odor, even when no other food substance or fluid of any kind was provided them. After four days they were still alive, but greatly emaciated and their weight loss (average 6.4 gms.) was as great as that sustained by starved animals which received nothing but water. However, histological examination of the intestinal epithelium of these animals revealed that there was no general breakdown of the tissue, even though there was marked leucocytic infiltration. There was also some slight mitotic activity. Orcein staining of these tissues was, however, very aberrant. It was not selective for nuclear structures, but was dispersed through the tissue in a hazy and indefinite manner, which rendered mitotic counts very difficult. For this reason the counts on these individuals are not presented in tabular form, and also because the amount of Amigen received by each mouse is not known, although certainly it was very minute in each case.

Since this method of administering Amigen resulted so inconclusively, we resorted to forced oral feeding, 0.1 cc. being administered

*Threonine, 3.1%; Valine, 5.3%; Leucine, 9.6%; Isoleucine, 4.9%; Lysine, 5.3%; Tryptophane, 1.4%; Phenylalanine, 4.1%; Methionine, 2.7%; Histidine, 2.0%; Arginine, 2.4%. The solution is made up in 5% dextrose, adjusted to pH 6.4.

hourly until 0.5 cc. daily was consumed. This experiment, like those previously described, was conducted for a four-day period on normal young mice, 50 days of age. The average number of dividing cells per field for the epithelium of the orally-fed mice appears also in Table II, and is as great, and sometimes in excess of, the numbers obtained in controls, even though there was some loss of weight in each animal. The histological picture was entirely normal, though there was some hazy dispersion of the orcein stain in preparations from this experiment.

It should be noted that the amount of Amigen administered was entirely empirical, since we had had no previous experience with its use. The amount employed was proportionate to that suggested as the *minimum* human dose. It is entirely possible that normal weight could be maintained by appropriate adjustment of the dosage, or by supplementation with fluid fats. Despite the loss of weight, however, both milk-fed and Amigen-fed mice were capable of producing dividing cells in a number comparable, or slightly superior to, controls. There does not, therefore, appear to be any relationship between weight gain or loss and number of mitoses.

The experiments just described appear to point to the apparent impossibility of suppressing mitotic activity in mouse intestinal epithelium by means of fluid diet unless the animals are actually on the point of starvation. This took four to five days in our mice. Attention may be called here to Macklin's statement (2) concerning the time mice will live without feeding, which is apparently based on the observations of Sun (3) who found that albino mice would not live without food more than 64 hours in summer and 24 hours in winter. That we have been able to maintain mice for much longer times without feeding is difficult to explain, unless our pre-experimental diet affords more adequate preparatory nutrition than was provided in Sun's experiments. His animals lost 20-25% of body weight in the 24 to 40 hours' starvation, and he reports that animals with a body weight of 11-13 grams were more resistant to starvation than animals above 14 or below 10 grams. All of our animals weighed greatly in excess of these amounts at the onset of the experiments, and yet survived anywhere from two to four times as long. No data are given by Sun concerning the age of the mice used in his experiments.

Our histological observations are nevertheless in complete agreement with those of Sun, at least so far as the starved animals are

concerned. He writes: "Starvation causes degenerative changes in the apices of the intestinal villi," and "in advanced starvation the terminal cells of the villi become a shapeless mass." His figs. 1-5 are drawings showing the comparison between normal intestinal epithelium and that of mice starved for 24 and 40 hours respectively. Our plates I and II show photographically the influence of our diets on numbers of dividing cells in the intestinal villi and on general maintenance of histological structure. Figs. 1 and 5 and text fig. 1 show the normal intestinal epithelium with large numbers of mitotic figures; figs. 2 and 6 indicate that little change occurs when animals are maintained on milk; the remainder of the photographs show the gradual loss of mitotic activity with progressive starvation. The final stage, taken from an animal immediately after death induced by four days' starvation (figs. 4 and 8) shows complete loss of structure of the intestinal villi, and there are no mitotic figures whatsoever.

No attempt was made in our experiments to re-feed partially starved animals nor to note the course of repair of the degenerated tissues. According to Sun's observations, villi become almost normal in appearance after 4-10 hours of refeeding, which agrees with the statement on p. 301 of Jackson's treatise (4) on the effects of inanition and malnutrition: "Upon refeeding after a period of inanition, mitosis (which is depressed during inanition) is greatly accelerated in the cells of the various atrophic intestinal tissues and the normal weight and structure may be promptly restored. . . . In experimental inanition, the degenerative lesions in extreme cases may render recovery impossible." A more recent paper by Grant (5) reports that in cats the surface epithelial cells of the digestive tract can be replaced within a few hours.

Although our initial purpose of suppressing mitotic activity in intestinal epithelium by means of fluid diet was not accomplished, the experiments are nevertheless presented because of their suggested application to diet therapy of enteric lesions. The medical literature is filled with discussions of the relative efficacy of various diets in the treatment of gastric and duodenal ulcers. Our results would seem to support the use of rich fluid diets and perhaps also of protein hydrolyzates to afford a non-abrasive source of nutrition which at the same time produces rapid cell division and consequent healing.

The authors wish to express their thanks to Dr. Mary A. Bennett of the Institute staff for helpful suggestions with respect to synthetic diets.

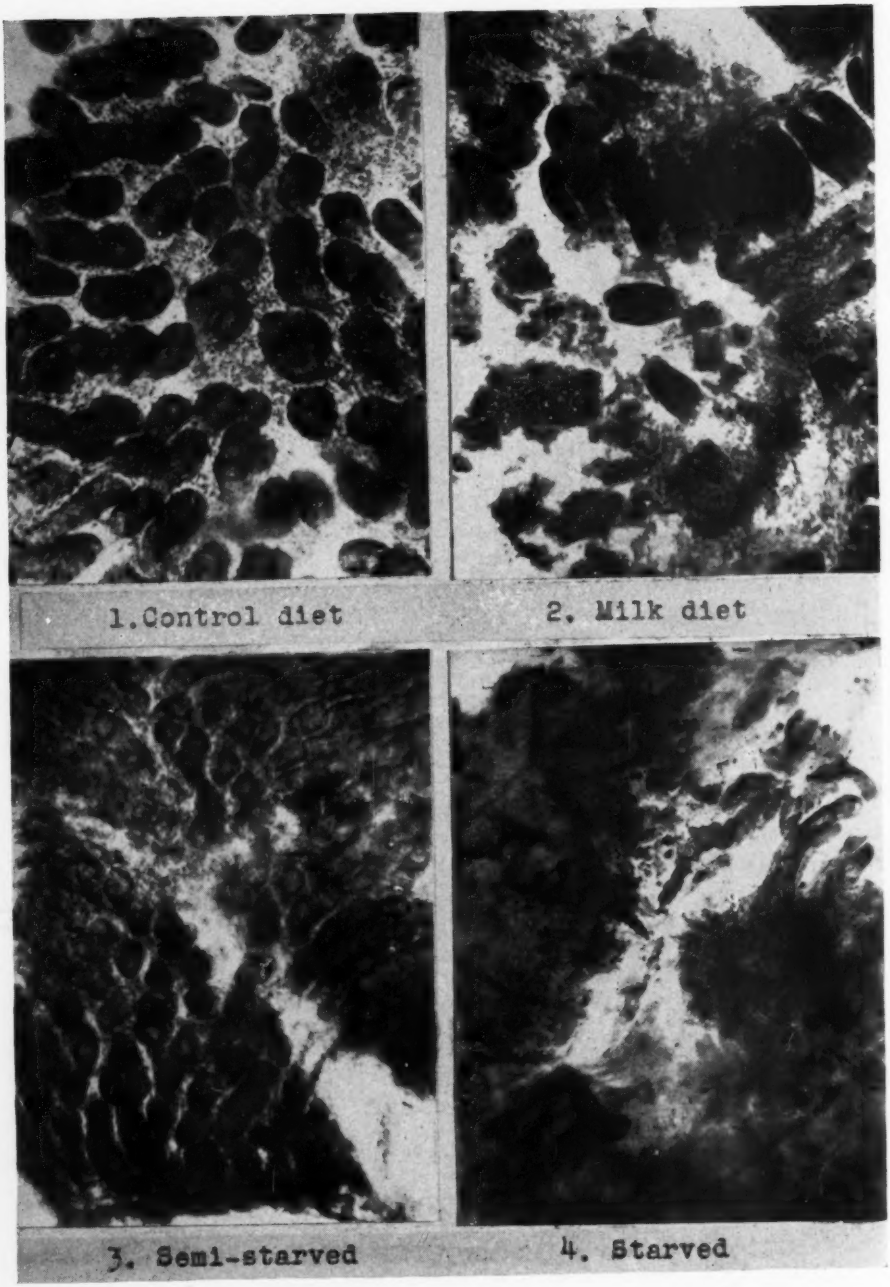

1. Control diet
2. Milk diet
3. Semi-starved
4. Starved

PLATE I

Figs. 1-4 are photographs of acetic-orcein smears of intestinal epithelium of animals numbered 340, 336, 331 and 332, respectively (Cf. Table I). Magnification 80X.

1. Animal fed on control diet of grains and compressed foods.
2. Animal fed four days on milk. Numerous mitotic figures.
3. Animal given water only for four days. Note decrease in size and shortening of intestinal villi, with withdrawal of substance from core.
4. The same (water only) showing total degeneration of villi in an animal dead on the fourth day.

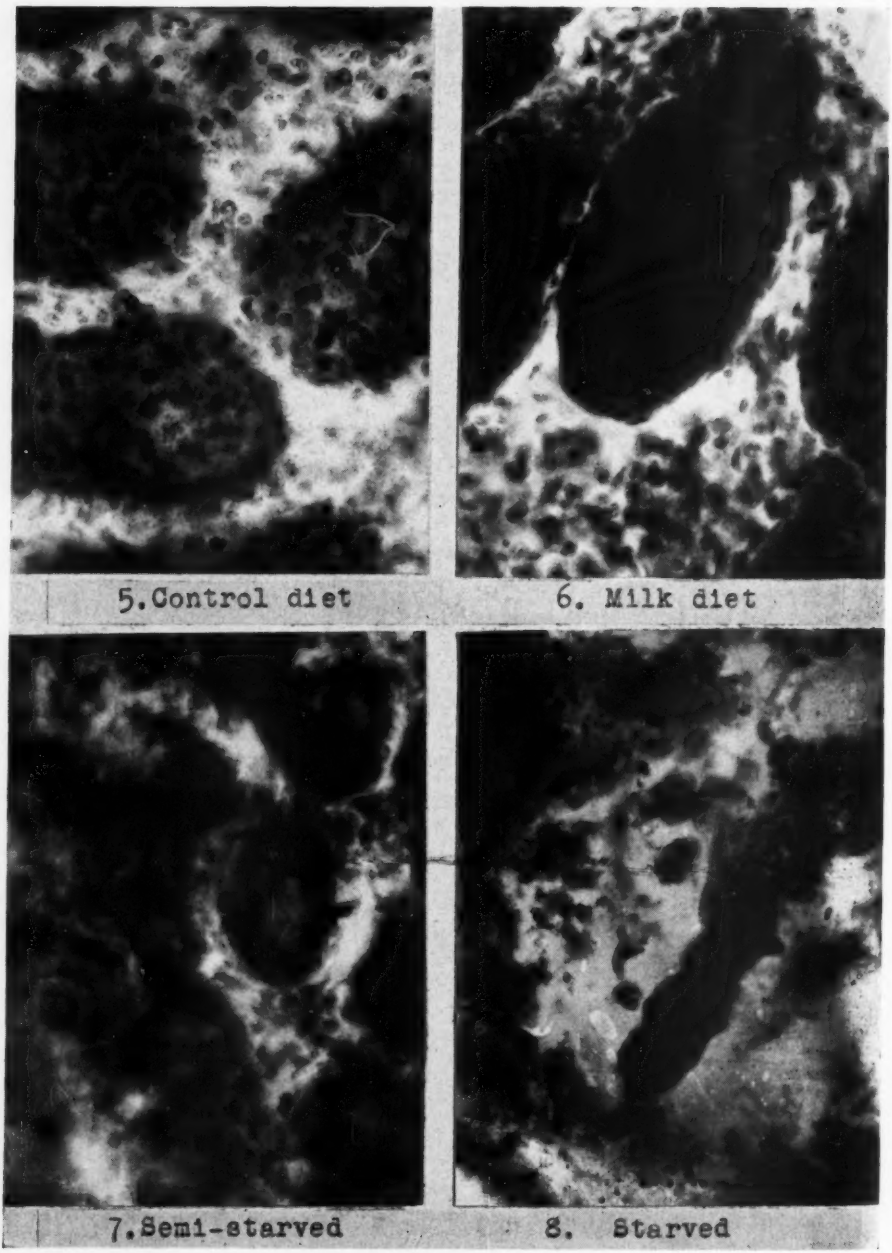


PLATE II

Figs. 5-8 inclusive. The same materials as shown in figs. 1-4, respectively, x 360.

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